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# Off-Flavors in Aquaculture

EDITED BY

**Agnes M. Rimando**
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# Off-Flavors in Aquaculture

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# Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

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# Preface

Aquaculture is the production of aquatic organisms under controlled conditions and is a rapidly growing segment of agriculture attributable to increasing human population and the desire for healthier food items. Attempts to meet the increasing demands for aquaculture products have been hindered by unmarketable aquaculture products caused by *off-flavor*. Off-flavor is most commonly associated with specific cyanobacteria (blue-green algae) growing in the water systems used for aquaculture. Control of these off-flavor compound-producing microbes is difficult and an ideal management approach has not yet been achieved. Challenged with this problem, scientists have engaged in a variety of research approaches to help aquaculturists manage flavor-related problems. In a previous scientific meeting (special session entitled "Management of Blue-green Algae and Off-flavor" held at the 1994 World Aquaculture Society meeting, New Orleans, Louisiana), scientists presented their research findings. This special session focused mainly on off-flavors in catfish aquaculture and the proceedings from the symposium were never published collectively in a book.

This American Chemical Society (ACS) Symposium Series volume is based on a symposium entitled *Off-Flavors in Aquaculture* that took place at the ACS 223rd National Meeting on April 7–11, 2002, in Orlando, Florida. This symposium is the first and most comprehensive one ever held to date and encompasses many types of aquaculture worldwide. This book includes 17 chapters covering topics such as the following: (1) the physiological attributes of cyanobacteria and the environmental conditions that favor their dominance of phytoplankton communities in aquaculture ponds; (2) approaches to control noxious cyanobacteria using synthetic compounds, naturally occurring secondary metabolites, and by biological means; (3) analytical methods for the quantification of off-flavor compounds; (4) storage sites of off-flavor compounds in fish; and (5) the economic impact of off-flavor on catfish aquaculture, the largest and fastest growing type of aquaculture in the United States. Included are chapters discussing off-flavor in catfish, shrimp, salmon, Nile tilapia, blue mussels, trout, and sea urchin gonads. Several recent technological advances concerning management of off-flavor are reported.

We believe that this book will serve as a valuable information source for scientists and aquaculturists and will also be an important reference book for students interested in the field of aquaculture.

We thank the symposium participants and all of the authors who contributed to this book. We are grateful to the ACS Division of Agricultural and Food Chemistry for providing financial assistance and a forum for this symposium. We are also thankful to the reviewers for their valuable critique of these chapters.

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## Chapter 1

# Off-Flavors in Aquaculture: An Overview

**Kevin K. Schrader and Agnes M. Rimando**

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The demand for aquaculture products continues to accelerate worldwide. One of the most economically significant problems encountered in aquaculture is "off-flavor" in the cultured product. Off-flavors can be related to the diet of the cultured animal, caused by inadequate post-harvest management strategies, and/or environmentally derived. The latter has received the most attention in terms of research approaches for developing methods to prevent off-flavors in aquaculture products. The production of off-flavor compounds by certain species of cyanobacteria is the greatest contributing factor to off-flavor in fishery products. In this chapter, the types and causes of off-flavor that may occur in a wide variety of aquaculture products are discussed. In addition, the most recent developments in research for the prevention of off-flavor in aquaculture are discussed, and recent technological advances in off-flavor detection are presented.

Within the last half century, there has been a great increase in the demand for aquaculture (farmed) products worldwide due to the rapid growth of the human population and excessive harvesting and overexploitation of fish and other aquatic animals from the world's oceans. Also, the demand for fishery

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products has increased due to their recognition by consumers as a "healthier" food item compared to other meat products. Aquaculture, the rearing of fish and other aquatic animals, provides the best approach for meeting this increased demand. Currently, aquaculture provides approximately one-third of the world's fishery products. Asia produces most of the world's supply of farm-raised fish. The United States is ranked tenth in the world in cultured fish production, and aquaculture is the fastest growing type of agriculture in the United States (*1*). The largest and fastest growing segment of aquaculture in the United States is farm-raised catfish which are produced mostly in the Mississippi Delta.

One of the many challenges facing aquaculture is producing and maintaining a quality, good-tasting product. The flavor quality of cultured fishery products can be affected by diet (food source, quality, and composition), flavors derived from the environment, and post-harvest handling and storage practices. Typically, the objectionable odors or tastes detected in aquaculture products are referred to as "off-flavors." The most common off-flavors encountered in aquaculture are due to the absorption of odorous compounds from the water. Off-flavor problems cause inconsistent product quality that may adversely affect consumer demand and, ultimately, hinder industry development and decrease profits for producers and processors. In addition, off-flavor problems can directly increase production costs for producers. In the United States catfish industry, off-flavor problems were calculated to have increased production costs by as much as U.S. $47 million in 1999 (*2*).

Although aquaculture occurs worldwide, the economic impact of off-flavor problems has been studied most extensively only in catfish aquaculture in the southeastern United States (see Chapter 2). Economic losses occur not only at the farm level but also at the industry level. At the production level, economic losses due to off-flavor range from U.S. $0.04 to U.S. $0.26 per kg of catfish (*3*, *4*). Societal costs of off-flavor problems in catfish production have been estimated to be equivalent to 12% of the annual revenue received by catfish farmers (*5*). Poor flavor quality of cultured catfish reduces profits for the entire industry due to decreased market demand.

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## Diet (Natural and/or Manufactured)

The main route of absorption of many waterborne off-flavor compounds by fish is across the gills and/or skin (*6*, *7*). Ingestion of the compounds during the consumption of food and water provides another possible route of uptake of off-flavor compounds since these compounds can be absorbed across the lining of the gastrointestinal tract (*6*, *7*). Preventing the consumption of natural food sources available in the aquatic environment in which the cultured organisms are being reared can be difficult. For example, catfish that develop "rotten-fish" off-flavors may have consumed dead, decaying fish as they forage for food (*8*). Also, fish may inadvertently ingest certain types of microorganisms that contain off-flavor compounds while they are consuming feed or other sources of food.

The use and application of a manufactured, formulated feed in the rearing of fish and other aquatic animals permits better control of the flavor quality of the cultured product. In Atlantic salmon (*Salmo salar*), rancidity and off-flavor was higher in those fed on diets consisting of high levels of n-3 polyunsaturated fatty acids (PUFA) and vitamin E ($\alpha$-tocopherol) (*9*). A more thorough review of the effects of tocopherols on the flavor of Atlantic salmon is available (*10*). In sea urchins, the amino acid content of the artificial diet that they are fed will directly affect the flavor quality of the sea urchin gonad (*11*). The type of artificial feed used (e.g., algal diet compared to grain-based diet) will also affect the flavor quality of sea urchin gonads (see Chapter 7). Manufactured feeds containing high levels of marine fish oil can result in a "fishy" flavor in fillets of normally mild-flavored, farm-raised channel catfish (*Ictalurus punctatus*) (*12, 13*).

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## Environmentally Derived Off-flavors

There are many types of off-flavors that can be encountered in aquaculture products. Flavor descriptors are used to describe or characterize such off-flavors. Some of the more commonly encountered off-flavors are "earthy," "musty," "woody," "fishy," "rancid," "rotten," and "petroleum." A more complete list of the terms used to describe types of off-flavors is available (*14*). A "petroleum" off-flavor is typically associated with anthropogenic pollution of the aquatic ecosystem in which the cultured product is being farmed. Petroleum spills and effluents from petroleum refineries can contaminate fish, such as salmon raised in offshore pens (*15, 16*; see Chapter 8) and shellfish beds (*16-20*; see Chapter 6). Off-flavors related to the discharge of chemicals from pulp mills can also occur (*21, 22*). The flavors associated with pulp mill effluents have been described as "sewage" (*21*) and "phenolic" or "sulfide" (*23*). Additional information pertaining to water pollutants and their roles in fish off-flavors are available (*14, 24-26*).

Most off-flavors in aquaculture caused by humankind pollution are less common than those associated with microbes. The most commonly encountered off-flavor metabolites produced by microorganisms are geosmin [*trans*-1,10,-dimethyl-*trans*-(9)-decalol] and 2-methylisoborneol (*exo*-1,2,7,7-tetramethyl-[2.2.1]heptan-2-ol), and these compounds are attributed with having an "earthy" and "musty" odor, respectively. In aquaculture systems, earthy and musty off-flavors have been detected in Atlantic salmon (*27*), common carp (*28*), catfish (*6, 29-31*; see Chapter 3), shrimp (*32*; see Chapter 4), tilapia (*Oreochromis* *aureus* and *Oreochromis nilotica*) (*33, 34*; see Chapter 17), and trout (*35, 36*; see Chapter 5). Each compound is easily detectable in fish by humans. The sensory threshold concentration (THC) (the lowest concentration that can be detected by humans) differs between species of fish. For geosmin, the THC in fish is 6-10 μg/kg (*35, 37*). The THC for 2-methylisoborneol is 0.6 μg/kg in rainbow trout (*37*) and 0.7 μg/kg in channel catfish (*38*).

Certain species of actinomycetes (filamentous bacteria), cyanobacteria (blue-green algae), and fungi produce geosmin and 2-methylisoborneol. Actinomycetes and cyanobacteria can be found in many of the aquatic habitats used in aquaculture. Most of the actinomycetes that have been identified as producers of geosmin and 2-methylisoborneol are *Streptomyces* species (*39-56*). Actinomycetes are more numerous in the sediments of lakes and ponds than in the water column (*51, 57-60*) and are believed to be metabolically inactive in these sediments (*60, 61*). Within the last ten years, it has become generally accepted that odor-producing species of planktonic cyanobacteria are the major cause of earthy and musty off-flavors in fish aquaculture. Cyanobacteria are the dominant type of phytoplankton found in the water of catfish aquaculture ponds due to several physiological attributes that provide them with a competitive advantage (*62*; see Chapter 9). The cyanobacteria species that have been associated with geosmin-related off-flavor in catfish aquaculture are *Anabaena*, *Aphanizomenon*, and *Lyngbya* (*51, 63*). In trout farming systems in the United Kingdom, a geosmin-producing species of *Oscillatoria* has been associated with an earthy taint in cultured trout (see Chapter 5). Species of *Aphanizomenon*, *Oscillatoria*, and *Pseudanabaena* from catfish aquaculture ponds have been found to produce 2-methylisoborneol (*64, 65*). *Oscillatoria perornata* (Skuja) produces 2-methylisoborneol (*64*) and is most commonly associated with musty off-flavor in farm-raised catfish in Mississippi (*63*). This organism was tentatively described as *Oscillatoria* cf. *chalybea* (*64*), but was re-designated as *Oscillatoria perornata* (*66*) based upon the description by Skuja (*67*). Geosmin and 2-methylisoborneol are also responsible for many of the off-flavor episodes/problems in municipal drinking water systems worldwide.

There are additional odorous compounds that have been isolated from cyanobacteria and eukaryotic algae (*14*). One of these compounds, β-cyclocitral, is described as having a "woody" or "tobacco" odor (*68-70*). β-Cyclocitral has been isolated from cultures of *Microcystis* spp. (*71*), and some of these species can form blooms in catfish ponds (*12*). Although β-cyclocitral has been detected in the flesh of farm-raised catfish (*72*), it has not yet been established as a major contributor to off-flavor problems in farm-raised catfish.

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## Management and Prevention Strategies of Off-flavors from Cyanobacteria

Because the most frequently occurring off-flavors in aquaculture have been linked to the presence of odor-producing cyanobacteria, the main focus of scientists and aquaculturists has been to discover or develop methods to control the growth of these noxious cyanobacteria. Some of the approaches used for controlling cyanobacteria in catfish aquaculture have the potential for being used in other types of fish aquaculture. The use of algicides to kill or prevent the growth of cyanobacteria in aquaculture systems has received considerable attention over the years. Several types of synthetic compounds have been used as algicides in fish aquaculture including simazine and copper sulfate (*73-79*). Repeated, low-dose application of copper sulfate to fish ponds appears to be a promising management approach that is economical for producers (*77-79*; see Chapters 2 and 10). Natural compounds provide another source for selective algicides for use in aquaculture (*80-83*; see Chapters 13 and 14). Other approaches that have been tried for controlling cyanobacteria are the use of plankton-feeding fish (*84, 85*; see Chapter 12), the use of mussels in a Partitioned Aquaculture System (*86*), dyes to reduce light penetration into the pond water (*87, 88*), manipulation of nutrient levels in the pond water (*89-91*), and biological control methods (*92*; see Chapter 11). In 1999, the herbicide diuron was approved by the United States Environmental Protection Agency (USEPA) under a Section 18 Emergency Exemption for use in controlling off-flavor problems in farm-raised channel catfish (*2*). Diuron is toxic to *Oscillatoria perornata* at low concentrations (*93-95*) and has helped decrease off-flavor problems in Mississippi catfish production (*2*). However, future approval by the USEPA of diuron for use as an algicide in catfish production remains uncertain.

Once a contaminating bloom of cyanobacteria has been removed from a pond, the fish can become "on-flavor" due to purging of the off-flavor compound. The rate of elimination of the compound can be dependent upon several factors including water temperature, type of off-flavor compound, and fat content of the cultured animal (*31, 96, 97*). Some aquaculturists transfer off-flavored animals to an aquatic system that is free of contaminating compounds so that depuration of the flavors can occur.

## Post-harvest Management Strategies

As mentioned earlier, the delayed harvest of off-flavored animals until they are deemed to be on-flavor causes direct economic losses to the aquaculturist. For example, in catfish aquaculture, economic losses can be incurred while holding market-size catfish for the following reasons: 1) loss of catfish due to disease, poor water quality, and bird depredation; 2) additional feed costs; and 3) loss of income from fish not sold and from subsequent delays in restocking the pond with new catfish (*4, 14*). In rare cases, aquaculturists may be able to sell the off-flavor product to processors if the processor elects to utilize post-harvest methods to reduce or "cover" the off-flavor of the cultured product. One post-harvest strategy to mitigate off-flavor involves flavoring the fish fillets in a manner so that the off-flavor taint is undetectable or much less objectionable to the consumer (*34, 98-101*; see Chapter 17).

Proper handling, processing, and storage (preservation) methods are essential to help maintain the flavor quality of aquaculture products before they reach the consumer (see Chapter 16). The type of preservation method used (e.g., canning, freezing, freeze drying, salting) can influence flavor quality (*102-105*; see Chapter 17). Processing methods may include the application of food additives such as antimicrobial agents, antioxidants, cryoprotectants, and flavor enhancers (*102*). Flavor enhancement can be accomplished by "smoking" the cultured products which also helps mask off-flavors (*98, 106, 107*). A more thorough review of the effects of processing methods on the flavor quality of aquaculture products is available (*102*).

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## Analysis and Evaluation of Off-flavors in Aquaculture Products

Typically, aquaculture products ready for harvest undergo organoleptic analysis in which the products are "taste-tested" by trained individuals before being accepted by the processor. The human olfactory system (flavor is experience in the nose as well as the mouth) is very sensitive to many off-flavor compounds and is currently the best method available for detecting the wide range of off-flavors that could be encountered in an aquaculture product. In the catfish industry, there are several flavor classification schemes that have been developed to help trained sensory individuals at processing plants in the identification of flavors and determination of acceptable flavored catfish for processing (*8, 108-112*).

Instrumental analyses used for the identification and quantitative determination of off-flavor compounds relies primarily on gas chromatography-mass spectrometry (GC-MS). The sample preparation steps used in previous techniques for detecting off-flavor compounds in aquaculture products are time-consuming due to lengthy extraction and concentration steps (*32, 35, 96, 113-116*). Recent advances in GC-MS technology have permitted the development of more rapid techniques for detecting certain off-flavor compounds in aquaculture products. Methods utilizing solid-phase microextraction (SPME) and GC-MS along with microwave distillation have been developed for detecting geosmin and 2-methylisoborneol in catfish tissues (*117-120*; see

Chapter 15). So far, these methods have been most useful and beneficial for research purposes. Another type of analytical equipment with potential use in off-flavor compound analysis is the "electronic nose" sensor. Several studies have been performed that describe the manner in which this device has been used in evaluating fish freshness (*121-123*). More recently, a direct extraction method similar to SPME and referred to as stir-bar sorptive extraction (SBSE) has been developed and is 54X and 10X more sensitive than SPME in the detection of MIB and geosmin in water, respectively (*124*). While the new technique has not yet been used in the analysis of off-flavor compounds in aquaculture products, it is certainly applicable for this purpose. Several additional methods that have been developed for the detection of MIB and geosmin in water samples and that could eventually be used in aquaculture include the adsorption of MIB and geosmin onto a hydroxynapthamide cyclodextrin derivative preceding fluorometric analysis (*125*), immunoassay (*126*), and luminescent-type biosensor technologies (*127*).

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## Chapter 2

# Economic Impact of Off-Flavor to the U.S. Catfish Industry

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Off-flavor is a considerable economic burden to the U.S. catfish industry. Off-flavored catfish may cause consumers to reduce consumption or switch to another fish or meat product. Processors have higher costs due to off-flavor testing, and an uncertain supply of on-flavor fish to the processor increases costs and decreases operating efficiency. At the producer level, off-flavor has been estimated to add $15 to $23 million annually to catfish production costs. Various studies have estimated additional production costs caused by off-flavor harvest delays between $0.01 to $0.25 per kilogram of fish produced. Copper sulfate, other copper products, and diuron (subject to annual USEPA approval) are products that can reduce off-flavor occurrences when applied in low does over prolonged time periods. Diuron is the least expensive chemical to apply, followed by copper sulfate; and liquid copper products are the most expensive. Though progress is being made in controlling off-flavor, it remains a problem adding significant economic costs to the U.S. catfish industry.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

## Introduction

From the early years of the U.S. catfish industry until the present, off-flavor has been and continues to be one of the industry's most serious problems *(1-8)*. Consequences of off-flavor are numerous, expensive to address, and impact catfish consumers, processors, and producers. Additional production costs attributed to off-flavor in the U.S. farm-raised catfish industry ranged from $15 to $23 million dollars annually over the 1997-1999 period (9).

Farm level off-flavor problems can be carried over to the marketplace. Inconsistently flavored product can have potentially damaging effects on consumer confidence. For catfish processors, efforts to detect off-flavor are expensive and time-consuming, and, if undetected, can negatively impact sales. For catfish producers, off-flavor can delay market-sized fish harvests, increase production costs, and disrupt cash flow. It is difficult to control off-flavor because the offending algae occur naturally and can be found in most catfish production ponds.

This chapter will present research results focusing on economic impacts of off-flavor to consumers, processors and producers. More research has been conducted on producer level impacts than for impacts at the consumer or processor levels. This chapter will focus primarily on off-flavor effects at the catfish production level.

## Consumers

U.S. per capita consumption of fish and shellfish products was 7.07 kilograms (edible weight) in 2000, and per capita catfish consumption was approximately 0.5 kilograms of this total *(10)*. Total U.S catfish consumption in 2001 was approximately 136 million kilograms valued at $670 million. Catfish sales by U.S. regions were 43% to South Central states, 23% to Midwest states, 16% to Mountain Pacific states, 15% to South Atlantic states, 3% to Northeast states, and 0.2% to international markets in 1998 *(11)*.

Catfish consumers are the ultimate judges of fish taste. Their preferences are expressed in the market place through frequency and amount of purchases. Some consumers prefer catfish having a musty or earthy flavor, which is termed "off-flavor," but the majority of U.S. consumers prefer a mild, delicate flavored fish, which is termed "on-flavor." The harm of having off-flavored catfish in the marketplace is its effect on the consumer's quality expectation, i.e., the consumer expects to consistently receive a quality, mild-flavored product. Habitual consumers of catfish may not mind an occasional serving of off-flavored catfish, but if this occurs too often, consumers may decide to purchase

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catfish at another grocer or restaurant, at less frequent levels, not at all, or demand a discount for the uncertainty. The effect of off-flavored fish on new or infrequent catfish consumers may be more damaging. These consumers may come to an early conclusion that this is the expected taste and they may not try catfish again for a long time, if ever. This damages the catfish industry's reputation and future expansion.

A second impact of off-flavored catfish in the marketplace is that it raises the retail price to consumers. Because off-flavored fish cannot be sold, retail supply is less than firm supply, which drives up cost. This is a result of the marketed amount being less than actually produced *(5)*. Higher retail prices make catfish less competitive with other protein substitutes, such as other seafood, poultry, beef, and pork products.

## Processors

There are approximately 24 catfish processors in the U.S., with the majority of processing plants being located in the four major catfish producing states, i.e., Mississippi, Arkansas, Alabama, and Louisiana *(12)*. In 2001, these companies processed approximately 270,700 metric tons of catfish (round weight) into 134,200 metric tons of fresh and frozen product valued at $669 million. Frozen catfish products made up 59% of all processed fish sales and were valued at $404 million; the remaining fresh fish sales were valued at $265 million. Fillets made up 61% of all catfish sales, whole fish accounted for 21%, and the remaining 18% were comprised of nugget, strip, or "other" product forms.

Profit maximization for a specifically sized processing plant would be based on choosing the correct quantity of fish to be processed under a known capacity constraint. Off-flavored fish can create an uncertainty in the procurement of sufficient quantities of on-flavored fish and increase processing costs *(5)*. Additional costs arise from difficulties in scheduling individual pond harvests and the uncertainty of the daily quantity of fish arrivals. Optimal plant efficiency levels are based on known fish quantities being processed during eight-hour work shifts. Any reduction in fish quantity arriving could idle plant processing and result in reduced efficiencies and higher processing costs. As processors put more resources toward guaranteed delivery of optimal catfish quantities, overall cost of the final product will increase and may be passed on to producers, middlemen and consumers.

Another specific cost of off-flavor to the processor is the hiring of a fish flavor checker, that determines if catfish are on- or off-flavor and whether they are ready for harvest and processing. With variation in flavor quality, additional efforts are required by processors to control quality *(13)*. Catfish processors

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require farmers to bring fish samples in for a flavor check one week prior to a planned harvest and obtain tentative approval for purchase *(14)*. Fish are again checked the day before harvest and on the day of harvest to be certain off-flavor has not developed since the initial check. A final sample is taken from the transport truck upon arrival at the processing plant and before fish are unloaded. An off-flavor result at any sampling will delay the harvest until further tests confirm fish are on-flavor. Direct expenses for fish sampling and repeated trips to the processor are paid by the producer, not the processor.

In 1972, Lovell's research found over 50% of sampled catfish ponds were off-flavor *(15)*. Sindelar *(4)* worked with six processing plants comprising 80% of the U.S. catfish industry and found off-flavor restricted farm marketings by 9 to 33%. Elasticity of demand would determine whether this supply restriction would have a positive or negative impact on processor revenues. From Sindelar's derived demand function for catfish, an elastic demand at the farm level was estimated and implied a solution to the off-flavor problem would benefit processors and producers. Off-flavor is still a major factor limiting prompt harvesting of foodsize catfish. The average percent of off-flavor occurrences at four large processing plants in the delta region of Mississippi was estimated at 52% in 1997 and in 1998 and 43% in 1999 *(9)*. Even higher off-flavor occurrence rates routinely occur during summer months when conditions are optimal for growth of the offending algae.

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## Producers

In 2001 there were approximately 79,320 hectares of catfish production operated by 1,250 producers in more than 16 states. Approximately 270,700 metric tons of fish were produced at a value of $445 million (farm gate). Ninety-three percent of all catfish acreage is located in four south central states, with Mississippi having 56% of total acreage, Arkansas 18%, Alabama 12%, and Louisiana 7%. By point of first sale, direct producer sales to processors accounted for 94 percent of total foodsize catfish sales and remaining sales were for recreational use, to live haulers, or for retail, government, or other uses *(16)*. This section will cover the numerous economic consequences resulting from off-flavor delayed harvest of foodsize fish.

### How producers are affected by off-flavor

Efficiency in catfish production requires quick production and turnaround during the stocking, growing, harvesting, and restocking phases. Typically, the shorter the grow-out period the lower the fish mortality from disease, poor water quality, bird depredation, and other perils. While off-flavor does not harm catfish, it becomes an industry-wide problem when foodsize fish are ready for harvest, but are prevented from being harvested. Smaller pre-harvest sized catfish may have off-flavor, but because it doesn't affect growth, smaller fish are not tested. Thus, only market-sized fish are ever sampled for off-flavor. Producers of food-sized catfish are affected by off-flavor throughout the year, but more occurrences happen during warm weather months than in cooler months *(8, 17, 18)*. However, off-flavored catfish being held over the winter would likely maintain the flavor they have going into this period as cold water slows fish metabolism and off-flavor will not be purged as quickly as during the warmer months.

Off-flavor induced delays in harvesting catfish require producers to hold fish in inventory longer, resulting in additional feeding, labor, and other operational and fixed costs. Cash flow to the farmer can also be interrupted by delayed harvesting and can have serious financial consequences, such as not being able to meet short-, intermediate-, or long-term payments. Resulting fish can be larger than processors' desire and producers may receive a lower price. Delayed harvesting results in delayed restocking and reduces total fish produced over a longer time period. Prolonged holding of fish inventory can result in carrying fish through additional fall and spring seasons when pond-raised catfish are particularly susceptible to outbreaks of infectious diseases *(19)*. During the fall and spring disease periods, minor fish losses will occur and occasionally major losses occur. The producer bears the full cost of medicated feed used during disease periods, or if fish are taken off feed, producers bear the full cost of "lost" fish production, i.e., weight loss that will need to be gained back.

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## Duration of off-flavor episodes

The unknown duration of off-flavor episodes is another underlying problem having enormous implications to the catfish industry. Off-flavor can be acquired from a variety of sources, but most flavor problems in Mississippi pond-cultured catfish are caused by 2-methylisoborneol (MIB), a musty-smelling compound synthesized by blue-green algae; while in west Alabama, geosmin is the offending compound from blue-green algae *(7, 8, 14, 20, 21, 22, 23)*. Flavor problems caused by blue-green algae occur in episodes that coincide with the appearance and eventual disappearance of odor-producing species in the phytoplankton community. Populations of odor-producing blue-green algae are most common during the warmest months of the year, but the length of time that fish remain off-flavor varies considerably.

In 1990, Nerrie *(24)* reported that off-flavor and the lack of markets combined to force producers to carry 70% of all market-size fish through the winter months before they could be sold. Jolly and Engle *(25)* reported that carrying fish over the winter period added between $0.15 and $0.20 per kilogram to the production cost. Research conducted by van der Ploeg and Tucker *(26)* found off-flavor resulted in an additional 115 grow-out days before harvest could occur. The variation in length of time fish remained off-flavor varied considerably, ranging from 27 to 344 additional grow-out days in their 10-pond study. Tucker *(27)* reported off-flavor caused an additional 80 grow-out days beyond an acceptable harvest-sized catfish, with a range of 17 to 286 additional grow-out days in their 18-pond, 3-year experiment.

Through a mail survey of Mississippi catfish farmers, Hanson (9) estimated the number of additional production days caused by off-flavor to be 66 days per pond in 1997, 63 days per pond in 1998, and 48 days per pond during 1999. When extrapolated to the state level, the total number of additional production days resulting from off-flavor harvest delays was estimated at approximately 500,000 days per year. For 1999, the calculated cost of off-flavor to the Mississippi catfish industry was $14.7 million. On a cost per hectare basis, this amounted to off-flavor adding approximately $73 ($0.084/kg) or an additional 1% to variable production costs for each hectare of water surface area in operation.

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## Off-flavor induced costs to producers

As seen in Table I, several studies have been conducted that estimate the effect of off-flavor on catfish production costs. Keenum and Waldrop *(17)* estimated an industry-wide opportunity cost for off-flavor marketing constraints by applying an interest charge to the quantity of foodsize fish maintained in inventory because of off-flavor harvest delays. They specifically looked at off-flavor effects on catfish production during the first, second, third and/or fourth quarter of the year. The additional cost of holding fish to meet marketing constraints was estimated at $0.0386 per kilogram. For the first and fourth quarters of the year, they charged a 10% opportunity cost for delayed income on 25% of the fish held in inventory for 90 days. During the second and third quarters of the year, they applied the interest charge and an opportunity cost on foregone sales. The foregone sales represented the cost for delays in harvest, restocking, and new crop growth.

They accounted for additional value from fish weight gain during the holdover period and subtracted out additional feed costs. Additional value was greater than additional costs, but when interest and opportunity costs were included, the overall cost of production increased more than additional revenues. For the first or fourth quarter, off-flavor was estimated to increase production costs by $0.039 to $0.042 per kilogram. In their worst case scenario, off-flavor occurring in the second and fourth quarters, production costs increased by $0.079 per kilogram (for a total of $0.118 per kilogram when added to the marketing constraint cost of $0.039 per kilogram).

Another study *(28)* simulated the effects of off-flavor preventing harvests for 4, 8, or 16 weeks using a computer program called GROWCATS, developed by Killcreas *(29)*. Their simulation estimated additional per kilogram production costs of $0.01, $0.064, and $0.099, for an additional 4, 8, and 16 weeks of production, respectively. Annual net revenues for the 131-hectare simulated farm were reduced by $57,000 (59%) when the farm's 16 ponds had 16 weeks of off-flavor delays and annual net revenues were reduced by only $312 (0.3%) when only one of the 16 ponds had a 4-week off-flavor duration.

Engle, Pounds, and van der Ploeg *(18)* used multi-period programming techniques to analyze the effect of 13 off-flavor scenarios on commercial catfish net returns. Scenarios were developed using commercial farm data and off-flavor time patterns chosen to provide insights on the effect of off-flavor duration to production costs with and without cash flow considerations. They found eight of the 13 scenarios decreased net returns from the base scenario (no off-flavor) with no cash flow considerations. However, all eight optimal management scenarios used single-batch management strategies, which is in contrast to present industry practices. Also lower stocking rates were chosen in all feasible solutions, i.e., 9,800 fish stocked per hectare.

When cash flow was included into the mix, 12 scenarios decreased net returns, indicating how important real-life considerations are in modeling efforts. Farms having off-flavor 10 to 12 months of the year and farms having six consecutive months of off-flavor (October through March) could not meet their financial obligations. Feasible solutions that generated sufficient cash to make required quarterly payments involved multiple-batch management strategies. Also, some multiple-batch strategies stocked fish at higher rates, i.e., 14,800 fish per hectare, than did single-batch strategies. Overall, they estimated "lost revenues" resulting from off-flavor ranged between $0.05 and $0.06 per kilogram for scenarios with no cash flow considerations and between $0.04 and $0.25 per kilogram for scenarios considering cash flow. Since fish selling price varies by month, the month of sale affects net receipts. Based upon historical catfish price levels, sales in the months of June and October were critical to farm profits and delayed sales during these months adversely affected future harvest dates and net farm returns.

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**Table I. Catfish Production Cost Increases Related to Additional Holdover Periods Caused by Off-flavor Preventing Harvest**

| *Additional Cost of Production Due to Off-flavor, $/kg* | *Study* |
|---|---|
| $0.040 - $0.119 | Keenum and Waldrop *(17)*<br>- considered industry-wide marketing constraints, opportunity costs of held over fish, foregone sales and delayed restocking - new crop growth in their calculations |
| $0.011 (4 week delay)<br>$0.064 (8 week delay)<br>$0.099 (16 week delay) | Coats, Dillard, and Waldrop *(28)*<br>- used GROWCATS simulation model |
| $0.051 - $0.060 with no cash flow consideration<br>$0.040 - $0.249 with cash flow considerations | Engle, Pounds, and van der Ploeg *(18)*<br>- lowest and highest cost occurred when cash flow considerations were included<br>- cash flow injects realism into model |
| $0.088 - $0.198 | Hanson *(9)*<br>- data collected from mail survey of catfish farmers in Mississippi<br>- considered additional production costs and fish mortalities during off-flavor holdover period. |

Total 1999 estimated costs of off-flavor to the Mississippi catfish industry was $14.7 million dollars, down from an estimated $23 million in 1997 and 1998 *(9)*. These estimated costs were based on harvest delay time periods and costs incurred during additional production periods, i.e., operating expenses, value of fish lost, sampling transportation costs, and an opportunity cost for delayed sales income. Per kilogram estimated off-flavor costs were calculated for each year in their study. For 1999, when 167,800 metric tons of live weight catfish were produced in Mississippi, the additional off-flavor expense to the cost of production was estimated at $0.088 per kilogram. For 1997 and 1998, estimated additional off-flavor cost to production was $0.20 and $0.18 per kilogram of catfish produced, respectively.

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### *1. Operating expenses incurred because of off-flavor harvest delays*

From Hanson's *(9)* survey of Mississippi catfish farmers, operating cost estimates were calculated for additional feed, labor and other costs of maintaining fish until harvest could occur after off-flavor episodes. For 1999, the total additional production cost attributed to off-flavor was estimated at $195 per hectare. Breaking down the total into component costs, additional feed costs averaged $143 per hectare, labor cost $10 per hectare, and other expenses cost $42 per hectare. For the Mississippi industry level, total operating costs attributable to off-flavor harvest delays was $6.8 million in 1999, $10.5 million in 1998, and $11.4 million in 1997.

### *2. Fish mortality costs attributable to off-flavor holdover*

Negative net returns on catfish farms can occur when one or more ponds have delayed harvests. Catfish lost to disease, low dissolved oxygen, and bird predation during off-flavor-induced harvest delay periods was estimated to be $188 per hectare in 1999, $314 per hectare in 1998, and $274 per hectare in 1997 *(9)*. Disease was the most expensive loss category for each year, followed by poor water quality, and bird predation. For 1999, it was estimated that disease problems caused $114 per hectare in losses during the off-flavor holdover period. Catfish losses were estimated at $47 and $27 per hectare for poor water quality and bird predation, respectively, for the off-flavored induced harvest delays in 1999. Aggregate Mississippi level catfish losses from disease, poor water quality, and bird predation during off-flavor holdover periods was estimated at $6.5 million in 1999, $10.6 million in 1998, and $9.1 million in 1997.

Tucker *(27)* ran a 3-year off-flavor experiment and had two control ponds experience large fish losses from diseases that occurred during the off-flavor induced holdover periods. As seen in Figure 1, two control ponds had negative three-year average net returns due to prolonged off-flavor periods in which production costs increased and receipts were reduced from additional mortality. If off-flavor had been controlled, production harvests could have occurred on time and disease mortalities avoided.

### *3. Transportation cost to conduct off-flavor tests*

Producers also must bear the costs of additional pre-harvest fish sampling and transportation of samples to the processing plants for taste testing. Survey data analysis indicated that the average number of fish samples required before

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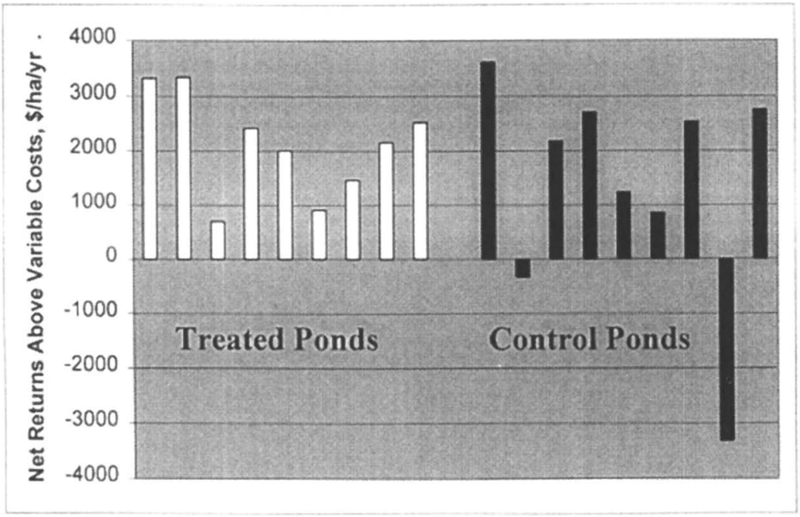

**Figure 1. Average 3-year Net Return Above Variable Costs from Copper Sulfate Treated and Control (No Treatment) Catfish Ponds**

a pond could be harvested was approximately 8 in 1999, 13 in 1998, and 14 in 1997 *(9)*. Producer transportation costs were calculated using round trip distances to the processor, number of samples taken and number of pond samples taken to the processor each trip. Average 1999 round trip mileage was 118 kilometers per trip. Total estimated off-flavor sample transportation costs for the state of Mississippi were estimated to be approximately $215,000 in 1999 or $6.15 per foodfish hectare; $246,000 in 1998 or $7.24 per hectare; and $304,000 in 1997 or $9.07 per hectare.

Delta and eastern Mississippi catfish farms were quite different, with delta farms generally ranging between 100- and 600-hectares and eastern farms ranging between 20- and 80-hectares. For 1999, estimated annual transportation costs associated with fish sampling were lower in the delta region at $3.66 per hectare than in the eastern Mississippi at $12.03 per hectare. The average number of samples taken to the processor before being declared on-flavor was higher in the delta region at 10.4 samples per pond than in the eastern region at 6.6 samples. However, farmers in the eastern region did not take as many fish samples per trip as they had fewer ponds per farm to sample each day and had to travel an average of 32 additional kilometers per trip than did delta farmers.

## Cost effectiveness of treatments to reduce off-flavor producing algae

Management of algae-derived off-flavors has proven to be an illusive goal because the catfish pond environment strongly favors the presence of blue-green algae. The most common management practice involves delaying harvest until the odor-producing algae naturally disappears from the pond and fish have the opportunity to purge the odorous compound from their flesh. This is an uncertain practice because changes in plankton community structure cannot be predicted and the process of purging may require variable lengths of time. Even though larger farms with many ponds may be able to manage off-flavor episodes by skipping harvest of the affected pond and harvesting fish from a pond with harvest-sized fish and acceptable flavor, this practice is not applicable on smaller farms with fewer ponds. As such, production and receipts on smaller farms can be more directly affected by off-flavor episodes. Engle *(18)* noted that the economic feasibility of an off-flavor removal system would have to be less than the additional production costs off-flavor caused to the producer and these costs have been estimated by various researchers and are provided in Table I.

In an attempt to avoid the burdens of delayed fish harvest due to unacceptable flavor quality, many producers attempt to eliminate odor-producing algae from ponds by using copper sulfate in a crystalline form, liquid copper-based product, or diuron. Copper-based algicides are the only family of

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algicides permanently registered for use in catfish ponds. Diuron has a temporary approval status and currently must be renewed annually through the U.S. Environmental Protection Agency. Table II provides a brief review of pertinent information on chemicals used to reduce off-flavor in the catfish industry.

Copper-based algicides have a long history of safe use in drinking water supplies, natural waters, and aquaculture ponds, but copper-based algicides are not ideal for blue-green algae control in fish ponds. The toxicity of copper products to algae and fish is controlled in a complex and poorly understood fashion by several water chemistry variables *(8)*. A consistently effective and safe treatment regimen for copper-based algicides is, therefore, not available.

Diuron is a substituted urea herbicide used for selective pre-emergence or early post-emergence control of seedling grasses and broad-leaved weeds in certain croplands. Diuron is a broad-spectrum herbicide with a 40-year history of safe use in terrestrial soils. The chemical is known to have algicidal properties at low concentration *(30)* and has several other characteristics that make it attractive as an algicidal candidate for off-flavor management *(31)*. These characteristics include a wide margin of safety between algicidal concentrations and concentrations toxic to fish and humans, freedom from complex interactions with other water chemistry variables, and lack of long-term persistence in the pond environment because the chemical is decomposed by natural microbiological activity. In addition, Novigen Sciences, Inc., *(32)* conducted a dietary exposure assessment and concluded that there was "... no chronic toxicological concerns from potential diuron residues in foods (p.17)."

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### *1. Cost effectiveness of copper sulfate in reducing off-flavor occurrences*

Tucker *(27)* conducted a three-year study to test the effectiveness of copper sulfate in curtailing off-flavor occurrences. In his research, there were nine copper sulfate treated ponds and nine control ponds receiving no chemical treatment. As seen in Table III, the average net return above variable costs for control ponds was $1,468 per hectare per year and was lower than the net return for ponds treated with copper sulfate ($1,941 per hectare per year). Detailed per pond receipts and costs of the major production inputs -- feed, fingerlings, aeration, and copper sulfate were presented. Standard deviations for net returns from control ponds were double those for returns from treated ponds. This increased variation was also seen for receipts, itemized variable costs, and total variable costs. As seen in Figure 1, the 3-year average net returns for nine copper sulfate-treated ponds had positive net returns whereas two of the nine control ponds had negative 3-year average net returns. The negative returns resulted from one control pond having delayed harvests each year of the study,

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**Table II. Comparison of Chemicals Used to Reduce Off-flavor in US Catfish Culture**

| *Chemical* | *Application Cost* | *Application Rate* | *Comments* |
|---|---|---|---|
| Copper sulfate<br>- Crystal form | From Tucker *(27)*:<br>- $ 7.06 per application<br>- Average cost = $156/ha/year | 5.6 kg/ha/week when water temperature is above 70 F | - Can be toxic to fish if over applied<br>- Results from Tucker et al., 2001 *(27)*:<br>- 50% reduction in all off-flavor episodes<br>- 75% reduction in methylisoborneol episodes<br>- 85% reduction in duration of off-flavor episodes<br>- 17% increase in aeration hours<br>- Increased levels of total ammonia and nitrite, but never to dangerous levels |
| Liquid Copper Products | | | |
| - Chelated copper algicide | Average cost = $311/ha/year | 2.34 liters/ha/week | - Uses less copper (only 20% of crystal copper sulfate amount)<br>- More convenient to use than crystal copper sulfate<br>- Expensive |
| - Chemically complexed liquid copper | Average cost = $623/ha/year | 4.68 liters/ha/week | - Uses less copper (only 20% of crystal copper sulfate amount)<br>- Longer persistence than crystal copper sulfate<br>- More expensive |
| Diuron | From Hanson *(9)*:<br>Average cost = $10.58/ha/year ($13.23/kg of diuron and 5.1 diuron treatments per pond per year in 1.3 meter deep ponds) | - 0.12 ml/m$^3$/week for up to 9 applications per year | - Non-toxic to fish<br>- Biodegrades over time, but slowly<br>- Accumulates in fish<br>- Uncertain legal future<br>- Not highly selective against noxious species<br>- From Hanson *(9)*:<br>- Farmers rate as moderately to highly effective<br>- Processor survey indicated reduction in percentage of non-acceptable fish since it was approved for use |

and from large losses of fish from disease during two of the off-flavored holdover periods. Reduced variation around the mean net return was interpreted as copper sulfate treatments stabilizing catfish production by reducing off-flavor induced harvest delays which, in turn, reduced the risk of fish losses to infectious diseases.

**Table III. Comparison of Catfish Enterprise Net Returns Above Variable Costs for Copper Sulfate Treated Ponds and Control (No Treatment) Ponds, $/hectare**

| *Item* | *Treated* | *Control* |
|---|---|---|
| Gross Receipts | 12,942 | 11,781 |
| Variable Costs | | |
| Fingerlings | 1,637 | 1,692 |
| Feed | 5,415 | 5,188 |
| Copper Sulfate | 157 | 0 |
| Aeration Electricity | 631 | 551 |
| Additional Variable Costs | 2,607 | 2,383 |
| Total Variable Costs | 10,447 | 9,814 |
| Net Returns Above Variable Costs, $/hectare/cycle | 2,495 | 1,967 |
| Number of Days in Production Cycle | 469 | 489 |
| Adjusted Annual Net Return, $/hectare/year | 1,941 | 1,468 |

Even with reduced variation in net returns from ponds treated with copper sulfate, the average of nine pond's net returns were negative for one year of the 3-year experiment. Control ponds (no treatment) had negative net returns for two of the three experiment years. Increased variation in net returns from control ponds was obvious, as the extreme values were both higher and lower than extreme values for copper sulfate-treated ponds.

Tucker conducted a break-even price analysis to determine the fish-selling price required to cover variable costs *(27)*. Results indicated that efforts to reduce off-flavor through copper sulfate applications were rewarded by lower average break-even prices. When no copper sulfate was applied, a breakeven price of $1.51 per kilogram was calculated compared to the lower $1.32 per kilogram for copper sulfate-treated ponds. Lower break-even prices indicated

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better performance because per kilogram returns above break-even prices indicate per kilogram profit.

### 2. *Cost effectiveness of diuron in reducing off-flavor occurrences*

The mail survey of Hanson provides detailed information on diuron usage, costs, and benefits to the Mississippi catfish industry *(9)*. In that survey, farmers were asked to rank the effectiveness of diuron in preventing off-flavor occurrences on their farm. Thirty-four percent gave diuron a ranking of eight on a scale of ten, with ten being "very effective" and one being "no effect." A cumulative 16% of all respondents gave a ranking of five (no difference) or lower (completely ineffective) to the diuron product.

Hanson conducted a benefit-cost analysis on diuron usage in the Mississippi catfish industry. The beneficial value of diuron was measured as the difference between the estimated annual aggregate off-flavor costs to the industry in 1999 minus its cost in 1998 or 1997. Costs were measured as the sum of diuron application costs and any fish mortality value stemming from diuron use. The diuron benefit-to-cost ratio was 42.5 to 1 when comparing 1999 to 1998 and 36.5 to 1 when comparing 1999 to 1997. Whenever the benefit-to-cost ratio is greater than one, benefits outweigh costs and the use of diuron was economically justified in its role of reducing off-flavor occurrences in the Mississippi catfish industry.

## Conclusions

The cost of off-flavor to the catfish industry is enormous. It has been estimated between $15 and $23 million annually between 1997 and 1999 (9). Various independent studies have estimated additional costs of production due to off-flavor delays to be between $0.01 - $0.25 per kilogram of catfish produced (9, 17, 18, 28). Considering a catfish production cost of $1.43 per kilogram, the additional off-flavor cost can be 3 - 17% of total production costs. Although average production and net returns are often used to evaluate catfish production systems, the results of Tucker's study *(27)* clearly indicate how monetary values can vary greatly from year-to-year and from pond-to-pond within a production year. Reducing the frequency of delayed harvests due to flavor problems reduces the risk of losing fish to infectious diseases, bird predation, poor water quality, or other problems that decrease overall fish production. Additionally, reducing off-flavor occurrences would reduce additional holdover operational and inventory costs. Management tools that

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reduce this variation will improve the prediction of production and input requirements, and thus allow better cash flow projections. Producers, lenders, processors, distributors, and consumers ultimately benefit from a more predictable catfish production system. Copper sulfate and diuron, when applied according to their protocols, can reduce off-flavor occurrences and stabilize economic performance of catfish production systems over systems using no chemical algicides.

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## Chapter 3

# Catfish Off-Flavors and Their Elimination

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Due to an increasing population, desire for seafood and over harvesting of natural resources, aquaculture has become a billion-dollar a year industry in the United States. Aquaculture is essentially the production of aquatic organisms under controlled conditions. Although conditions are controlled to a certain degree, absolute control of water quality is not possible in these environments. This reason alone is a huge problem that can cost the industry millions of dollars a year. In catfish, geosmin and 2-methylisoborneol are two compounds that are primarily responsible for imparting a musty/earthy off-flavor. These compounds are secondary metabolites of some species of blue-green algae and actinomycetous bacteria. Several pre-harvest and post-harvest techniques have been used in an attempt to eliminate this off-flavor problem in catfish. This chapter will cover the general catfish off-flavor issues and examples of methods for eliminating the off-flavors.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

## Catfish Aquaculture Industry

Due to an increasing human population, increased desire for seafood, and over-harvesting of the world's oceans, aquaculture has become a billion-dollar a year industry in the United States. Channel catfish farming is the largest U.S. aquaculture industry, with Mississippi, Arkansas, Alabama, and Louisiana as the leading producers accounting for 95% of the total U.S. sales (*1*). In 2001, production sales reached $443 million which represented a decrease of 12% from 2000. This decrease resulted in an increase in fish inventory with a 52% increase in large fish, which has implications for off-flavor issues. Farm-raised catfish processed in 2001 totaled 43.8 million pounds, an increase of 6% from the previous year (*1*). Of this processed fish, 21.6 million pounds were sold for $45.2 million with 61% sold as fillets. However, the problem of catfish off-flavor continues to threaten the prosperity of this business.

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## The Off-Flavor Issue

Off-flavor of catfish is a problem for the aquaculture industry in the southern part of the United States. Every year, many catfish are not marketable largely because of their musty/earthy off-flavor. Up to 80% of harvestable fish can be off-flavor during any one year (*2*). When catfish are off-flavor, they can not be marketed which results in increased production costs. Increased production costs have been estimated to be $0.10/kg for a 16-week off-flavor episode (*3*) or from $5.8 to $12 million annually (*4*). Because the fish can not be harvested, it potentially results in "downtime" at processing facilities. Also, if off-flavor fish makes it to market, consumers may perceive catfish as an unacceptable product.

### Cause of Off-Flavor

Aquaculture production requires the aquatic environment to be relatively stable. This stability has been attributed to the phytoplankton and bacterial communities that live within these ponds (*5*). Phytoplankton are single-celled organisms that produce oxygen as a byproduct of photosynthesis (*6*). In a commercial catfish production pond, the phytoplankton provide oxygen. They also help remove certain types of metabolic wastes. Although phytoplankton be beneficial to the pond ecosystem, they can also be a factor in making it undesirable for fish production.

Due to high fish stocking densities, aquaculture ponds receive large nutrient inputs from fish feeds and also from fish excretions, and sediment mineralization/resuspension (*7*). For example, it is common practice in an intensive catfish production system to stock a pond at 20,000 to 30,000 fish per hectare or approximately 1.6 to 2.5 fish per cubic meter (*8*). Unfortunately, because these pond systems are "static" systems and high amounts of nutrients are added daily, algal blooms and bacteria are encouraged to grow and proliferate(*6*).

Algal blooms mainly consist of blue-green algae (cyanobacteria) and form near the surface of the water restricting light penetration (*9*). Actinomycetes and blue-green algae are sources of off-flavor in aquatic systems. Actinomycetes are associated with the soil and examples of genera that typically produce earthy-muddy off-flavors are *Streptomyces* and *Nocardia*. Blue-green algae are the main source of the musty and muddy off-flavors found in aquaculture ponds (*10*). Genera of these blue-green algae that have been associated with earthy/musty off-flavors include *Anabaena*, *Aphanizomenon*, *Nostoc* and *Oscillatoria*.

Musty/earthy flavors are caused by algal formation of geosmin (GSM) and 2-methylisoborneol (2-MIB) (*4, 11*). GSM is mainly associated with *Anabaena* species, while 2-MIB is mainly associated with *Oscillatoria* (*12, 13*). In 2001, a study showed that *Anabaena spiralis* and the *Oscillatoria* species, *O. perornata* and *O. sancta,* were abundant cyanobacteria in Louisiana catfish ponds (*14*). These compounds have hydroxyl groups which provide dual solubility characteristics, and they are terpene compounds which imparts their volatile characteristic.

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## GSM and MIB Uptake and Distribution

GSM and MIB in the water of ponds can enter into catfish through ingestion of cyanobacterial cells, absorbtion through the skin, and most easily across the gills of the catfish and will tend to accumulate in the fatty tissue of the fish (2, *15, 16*). In catfish, the transport of the off-flavors through water and blood is increased due to the off-flavor compound structure and function (*17*). These off-flavor molecules are fat-soluble and likely to deposit themselves under the skin (*18*).

Dionigi et al (*15*) found a direct positive correlation between the fat content of catfish fillets and MIB concentration in the fillet. Fish with nore adipose tissue were observed to uptake more MIB than leaner fish (*16*). The fish that had greater than 2.5% muscle fat were considered to be "fat", and the fish that had greater than 2.5% muscle fat were considered to be "fat", and the fish that had 2% or lower muscle fat were considered to be "lean". Within a 24 hour period, the fat fish took in 3X more MIB than the lean fish. This study concluded that attempting to rear lean fish would be more profitable because they would have lower levels of off-flavor compounds and have an increased rate of purging (*16*).

Martin et al. (*19*) did a pharmokinetic study to determine tissue disposition of MIB in catfish, where 1 mg/kg body weight. MIB was intravascularly administered. Only 20% of the administered dose remained in the catfish tissues after 2 hours. The main area of MIB accumulation at this time was in the skin which included the subepidermal fat (*19*). After 24 hours, twice as much MIB was found in the epidermal fat than in the skin and greater than 20X the level found in the muscle. Levels of MIB decreased in all tissues with time. MIB was not found in the bile and only 6% was excreted in the urine. These results indicate that the MIB was either eliminated through the skin and gills or was rapidly biotransformed (*19*).

Higher water temperatures result in a more rapid uptake of MIB by catfish (*20, 21*). The rate that the off-flavor is absorbed depends also on the length of exposure time and the concentration of compounds in the water. When catfish are exposed to 1 ppb of geosmin at 20°C, geosmin was rapidly absorbed (*22*). Off-flavor in catfish develops within hours when they are exposed to high concentrations of MIB in water, while exposure for days to weeks is required for catfish to develop a detectable off-flavor in water with low MIB concentrations (*18*). The rate of uptake can also be affected by the species of fish and their physiological state (*23*). Off-flavors absorbed on or in the skin are usually removed from the fish when the skin mucousa is removed during the processing of the fish (*23*).

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## Sensory Evaluation and Odor Threshold

The use of instrumentation to detect flavor thresholds can often be expensive and complicated (*24*). The methods associated with quantitative chemical analysis are time-consuming and, although they are sensitive enough to detect off-flavors at low levels, it is not feasible to bring the instrumentation required out to the ponds. Newer, rapid methods using immunoassay are more feasible for field application, but are not commercially available yet. In cases where there are low levels or even when the causative compounds are unknown, it is best to use sensory evaluation (*24*). This method is widely used because low levels of odorous compounds can be detected, discriminated, and flavor intensity identified (*25*).

In order to prevent the harvesting of catfish with off-flavor, sensory evaluation has been used to detect levels of GSM and MIB in preharvest samples. Quality control practices may vary within the industry, however it is routine to collect samples frequently from ponds before harvesting them (*15*). Normally two or three fish from each pond are taken to a processing facility for preharvest flavor testing (*15, 25*). Experienced flavor checkers are able to detect from 0.1 ppb to 0.2 ppb MIB in catfish (*26*). If fish are deemed to be unacceptable, they are not harvested (*20*). Sensory evaluation for off-flavor in catfish suffers from an inadequate sampling procedure. It has been shown that the off-flavor in fish from ponds is heterogeneous in nature and this leads to sampling error since very few fish are being tested out of ponds containing as many as 10,000 fish per hectare (*15, 24*).

The consumer flavor threshold for geosmin in fish is 0.6 ppb to 6 ppb and 0.08 ppb to 0.6 ppb for MIB, depending on the species of fish (*27, 28*). For channel catfish, 0.7 ppb was determined to be an appropriate consumer threshold for MIB (*29*). Therefore, less than 0.7 ppb for each off-flavor compound in catfish can be used as a target for any elimination method.

## Elimination of Off-Flavors

Fish that are rejected due to off-flavor must be placed back into ponds until they are deemed "on-flavor". This practice results in greater production costs associated with longer feeding times as well as a possible exacerbated problem with increased off-flavor due to higher fat content of larger fish. Current technologies for assuring the flavor quality of catfish fillets have not been completely adequate. These procedures have included pre-harvest and post-harvest methods. The remainder of this chapter will cover the issues of some of the methods used, or which could be used, to minimize formation of off-flavors or eliminate them after off-flavor contamination of the fish.

### Pre-Harvest Methods

#### *Purging and Raceway Use*

Although off-flavor is easy to acquire, once these chemicals are present in fish it may take several days to bring them back to "on-flavor" (*30*). A common practice is to purge odorous chemicals out of fish. However, this method can be very costly and time-consuming. Purging is achieved by holding fish in a smaller pond and continuously flushing them with fresh water (free of off-flavor chemicals) until off-flavor is gone. MIB can usually be purged within 3-5 days. Geosmin is more difficult to purge and can take up to 3-4 weeks to be reduced below sensory detection levels (30). For these reasons, off-flavor compounds

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should be identified before choosing purging as a "recovery" method (*18*). During this process little to no fish feed may be applied, consequently fish often lose 1-5% of their body weight.

Studies have shown that the rate of purging is affected by water quality, holding conditions, water temperatures, concentration of odorous compounds in fish, and fat content of fish (*25*). A study performed by the United States Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, Louisiana, suggested that MIB and geosmin could be purged from catfish within 2-3 days provided that the fish are held in water free of off-flavor metabolites (*30*). Other studies have suggested that the best conditions for depuration of fish are those in which the fish are lean and the water temperatures are high (*16, 20*). In fish with 5% fat held at 34°C, the musty flavor of MIB can be purged within sixty hours to levels below the threshold of 0.7 ppb in fish initially containing 6 to 11 ppb (*20*). For fish held at 6.5°C with 15% fat, 140 hours were required to reach the threshold level. The advantages to using purging are that it is relatively simple and does not create an environmental issue. The disadvantages are that there are water and labor costs for moving the fish to fresh water. Also, fish could either lose weight if not fed, resulting in decreased returns, or gain weight if fed during purging that could result in fish that are too large to process easily. There are increased costs for feeding fish ready for harvest, and the time required for purging can result in downtime for processors. The water temperature is not easily manipulated to control the rate of purging. Finally, moving fish causes them to be stressed making them more susceptible to disease and water quality problems, which could result in a loss of fish through death and therefore decreased returns.

One way to minimize build-up of off-flavor and to minimize loss of fish due to moving them to ponds with fresh water is to use a raceway system with cages (*8*). In this system, the fish are contained in cages in one area of the pond which has raceways for water flow. The raceways help to minimize fish waste buildup, and there is an area that is deeper than the rest of the pond and that is far away from the cagged fish for settling of wastes. The water is constantly aerated mechanically in the area of the cages to maintain adequate oxygen levels. There are several advantages to this type of system, which has also been termed the partitioned aquaculture system (PAS) by researchers at Clemson University, South Carolina (*31*). It is easier to prevent animals, such as birds, from preying on the fish in the cages since the cages require less material (e.g., netting) for coverage than an entire pond. There are reduced labor costs for harvesting as well as for disease prevention due to being able to treat more fish in a more concentrated area with lower amounts of drugs (*31*). Another obvious advantage is that water will be conserved. Finally, algae-consuming organisms can be placed in or near the cages to minimize blue-green algae contamination. However, a disadvantage is that one of the most efficient algae-consuming organisms, tilapia, is not permitted for use in open aquaculture systems by law

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(*31*). Other disadvantages include an increase chance of disease transmittance due to the fish being in closer contact with each other, mechanical failure of the aeration system, and the capital costs involved in adopting this system.

### *Algicide Use*

The use of algicides is to kill the blue-green algae. However, killing these algal blooms may not improve the situation and could potentially make the problem worse in the near term. Treatment of ponds with algicide chemicals can result in beneficial algae also being killed. When algae die and decompose, oxygen is lost from the pond water and can result in suffocation and death of fish. When dead blue-green algae decompose, the cells lyse and release odorous metabolites into the water, which can still be absorbed by the fish (*22, 32*). A study by Zimba et al. (*33*) showed that diuron is an algicide that is selective to blue-green algae when applied at certain levels, and that it is quite effective in reducing MIB levels in water and fish fillets.

Some algicides may not be persistent long enough to be effective due to rapid dissipation, but if they are too persistent, the algicides can create an environmental concern (*34*). Also, some algicides, such as copper-based products, may be toxic to the fish if they are applied incorrectly. The toxicity of copper-based algicides towards blue-green algae depends upon water quality several factors such as pH, hardness, total alkalinity, and amount of organic matter (*18*). The advantages to using algicides are that they are easy to apply to ponds, they are relatively inexpensive, and it is easy to observe if they are effective in reducing blue-green algae blooms.

### *Biochemical Methods*

The objective of the use of biochemical methods is to either inhibit off-flavor synthesis by the algae or to enhance biotransformation of the off-flavors in the fish. These methods are experimental only and are not currently being used. Dionigi et al. (*35*) observed that certain compounds could inhibit the biosynthesis of geosmin in *Streptomyces tendae* bacteria. Treatment with N-octyl bicycloheptene dicarboximide reduced geosmin levels by 40% compared to untreated controls of the bacteria. This compound may have inhibited the cytochrome P450 mixed-function oxidases which may potentially catalyze the hydroxylation of a terpenoid precursor to form geosmin (*35*).

Schlenk (*36*) examined the relationship between the presence of MIB and P450 expression. Kidney and liver isoforms of P450 were induced when catfish were exposed to MIB and resulted in a 100% and 81% increase in the isoforms respectively. These results indicate that the isoforms may play a role in the

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biotransformation of MIB to more water-soluble metabolites to enhance ithe elimination of MIB (*36*). If such compounds could be added to feeds or applied in the same manner as an algicide to enhance biotransformation of off-flavors or prevent the blue-green algae from producing off-flavors, this method would be easy to use. The disadvantages to these types of methods are that it would be hard to determine the appropriate levels to use, there would be environmental issues since chemicals are being added to the ponds, and the costs associated with this method are completely unknown at this time.

## Post-Harvest Methods

### *Acid Treatment and Vacuum Tumbling*

Several studies related to preventing microbial growth on catfish fillets involved testing the sensory effects of acid treatment on fish fillets. Ingham (*37*) found that panelists could not distinguish between control and lactic acid-treated catfish fillets. However, Marshall and Kim (*38*) treated catfish fillets with acetic acid and/or lactic acid and observed that panelists preferred control samples over treated samples. Kim et al. (*39*) found that catfish fillets treated with lactic acid were less favorable in appearance and flavor compared to controls. Injection and dipping with antioxidants, such as ascorbic or citric acids, may not allow complete infusion at all sites of the fillet muscle and could result in adverse textural properties such as granular mouthfeel and chalky appearance as the acids decrease the pH (*40, 41*).

Forrester et al. (*42*) attempted to establish whether MIB found in catfish fillets post-harvest could be degraded after treatment with citric acid that was applied with vacuum tumbling. A 2% citric acid treatment resulted in a 36.8% loss of MIB, but consumers could not detect a difference in musty/earthy flavor compared to untreated controls. The failure to detect a difference was attributed to potential masking effects of the batter used to coat the fillets (*42*). There were no differences in texture detected instrumentally or by panelists. Consumers did detect sourness, a disadvantage of this method, in the treated samples and preferred the control to the 2% citric acid-treated samples (*42*). Another disadvantage is that vacuum tumbling requires special equipment. Advantages to using these acid treatment methods are that food grade chemicals can be used, the methods are easy to perform in a short time, and they are relatively inexpensive.

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### *Masking*

The purpose of this method is to cover-up the existing off-flavor in the catfish fillets. The advantages to using this method are that food-grade spices are used, the method is easy to use in a short time, and there may be the added benefit of the antimicrobial effects of certain antimicrobial spices. Some of the problems with using masking are that since consumers have variability in taste preferences, several spice formulations would have to be made. There also may not be a large market for the spiced product. In addition, the level of spice needed to mask the off-flavor may be excessive resulting in an unacceptable product. Smoking of the fish could also be used to mask off-flavor, but then it is not possible to have a fresh product. A study by Iredale and Shaykewich (*43*) observed that muddy flavor in trout could be minimized by smoking of the fish and the product was acceptable to consumers. Canning also reduced muddy flavor in trout, but the use of ribotide and citric acid could not mask the muddy flavor and actually resulted in a more intense muddy flavor than in the control (*43*). A 1993 study by Waagbo et al. (*44*) found that smoking of fish, in this case salmon, could mask off-flavors.

### *Ozonation*

Ozone is currently being investigated and is also being used as a replacement for chlorine-based chemicals for sanitation purposes in food processing, especially in the meat industry, and for water quality purposes, including bacterial, odor, and toxic compound degradation. Ozonation has been shown to improve the shelf-life of some products through microbial reduction without affecting quality, but some studies show that ozone can affect sensory quality of some products through lipid oxidation (*45*). Lag phases of microbial growth on whole fish were extended after storage of the fish on ice in a daily replaced atmosphere of ozone (*46*). There were also lower microbial counts during storage as compared to controls without ozone. Sensory quality scores were higher for whole fish stored in ozone atmosphere and the fish were more oxidatively stable than control fish as measured by thiobarbituric acid (TBA) value (*46*). Research on the ozonation of catfish fillets to destroy microorganisms showed that up to 1.8 ppm aqueous ozone treatment for 5 to 10 minutes did not result in lipid oxidation (TBA test) before or during storage for 2 to 6 days at 4°C (*47*). There was also no change in color of the fillets as measured by Hunter system. Chen et al. (*48*) found no mutagenicity by the Ames test in shrimp meat stored in 5 ppm aqueous ozone in a 2% saline solution for 2 hours, although there was a small decrease in bacterial counts without sterilization.

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Recently, ozone has been applied to the aquaculture area for bacteria control, disinfection purposes, and water quality (*49, 50*). Ozonation has shown potential gains in catfish shelf-life, ice quality production, and more efficient operations of water chillers in fish processing plants (*51*). The U.S. Food and Drug Administration (USFDA) originally affirmed ozone as generally-regarded-as-safe (GRAS) for use as a disinfectant in bottled water (*52*), but recently approved ozone as GRAS for broad disinfectant and sanitizing applications in food processing (*53*).

Ozonation has the potential for destroying off-flavors in catfish fillets, since it has been shown to be effective in the degradation of MIB and geosmin in drinking water (*54*). The use of ozonation to destroy GSM and MIB in catfish fillets is currently under study at the Louisiana State University Agricultural Center, Baton Rouge, Louisiana. Initial studies have found that introducing 3 % ozone into water containing 10 ppb GSM and MIB resulted in the degradation of GSM (by 54% after 20 minutes of treatment) and MIB (by 64% after 30 minutes of treatment) (*55*). A 10-minute ozonation catfish fillets, spiked at 5 ppb GSM and MIB on the surface of the flesh, resulted in MIB and GSM levels below the human threshold. A 10-minute ozone treatment of a 10 ppb surface-spiked sample resulted in average concentrations of MIB and GSM of 0.158 ppb and 0.118 ppb, respectively (*55*). In comparison with the unozonated spiked fillets, which were below the human threshold of 0.7 ppb, MIB was reduced by 14% and GSM was reduced by 2.5%. Ozonation for the removal of off-flavors from catfish shows promise.

This off-flavor elimination method would be easy to use and implement into the existing catfish processing system. The capital and operating costs are relatively low. The method is anticipated to only take minutes to perform and may be carried out in either a gaseous form or in a liquid form dissolved in water. The negative aspects of using ozone treatments include safety concerns for workers, limitation for ozone reaching all parts of the fillet muscle containing off-flavors due to poor diffusion, and, since ozone is a strong oxidizer, other compounds in the fish such as lipids and nutrients may also be oxidized. Further study is required to determine the optimal time and ozone concentration combination for destroying MIB and GSM in spiked catfish fillets and in naturally-occuring off-flavor samples.

## Summary

Although there have been many studies that have attempted to find a solution to the problem of catfish off-flavors, none have been completely successful, and therefore, more advances in this field need to be made. As stated earlier, aquaculture in the United States is annually a billion-dollar

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industry, half of which is comprised of the production of channel catfish. Therefore, it would be very lucrative to uncover a resolution to major off-flavor problems in channel catfish aquaculture.

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# Chapter 4

# Off-Flavor in Pond-Cultured Marine Shrimp

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Off-flavor is common in fish, but also occurs in penaeid shrimp. Off-flavor compounds are soluble in lipid and tend to concentrate in shrimp heads. Thus, off-flavor usually is not a major concern unless shrimp enter the "heads-on" market. As in fish, the main cause of off-flavor in shrimp is compounds produced by blue-green algae (cyanobacteria). Blue-green algae can be expected in shrimp ponds when salinity of water is below 5 or 10 parts per thousand (ppt). Shrimp farming has extended into low-salinity areas and into inland areas where salinities of 2 to 5 ppt are achieved by mixing saline well water or brine solution with freshwater. Blue-green algae can be as troublesome in inland shrimp ponds as in freshwater fishponds. Where salinity is higher, filamentous blue-green algae may be a major component of benthic algal communities ("lab-lab") which may occur on bottoms of ponds with shallow or clear water. Procedures for controlling off-flavor in shrimp have not been developed. The usual response is to flush water through ponds in an attempt to remove blue-green algae.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Off-flavor caused by odorous compounds excreted by blue-green algae and actinomycetes is a common problem in pond fish culture. It also can be troublesome in pond culture of marine shrimp. However, off-flavor has not been a common problem in shrimp culture, and relatively little information is available on the topic. Lovell and Broce (*1*) described off-flavor in shrimp after a processing plant in Georgia found that a shipment of pond-reared shrimp from Ecuador was unmarketable because of bad flavor. The shrimp contained high concentrations of geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) in tail muscle, and a panel of sensory evaluators found that the tails had the intense earthy-musty flavor caused by this compound. Geosmin also is a common cause of off-flavor in fish, and it is thought to originate mainly from blue-green algae (*2, 3, 4, 5*). Although there are some general references to the occurrence of off-flavor in marine shrimp culture (*5, 6, 7, 8*), no other works dealing specifically with this problem could be found.

The purpose of this report is to provide some observations on off-flavor made during many consultations related to shrimp farm water quality.

## Blue-green Algae in Shrimp Culture

Shrimp farmers in Latin America often determine the relative abundance of major algal taxa in ponds. Blue-green algae usually are present in the phytoplankton communities of shrimp ponds, but they seldom comprise more than a small percentage of the phytoplankton community where salinity is above 10 parts per thousand (ppt). Shrimp ponds are managed in much the same way as fishponds with inputs of fertilizers and feeds. As production levels increase, nutrient inputs increase and phytoplankton blooms develop. In low salinity water, blue-green algae may become the dominant species of phytoplankton. Blue-green algae such as *Oscillatoria*, *Anabaena*, and *Microcystis* often associated with off-flavor problems in freshwater fishponds also are found in shrimp ponds.

Blue-green algae seem to become dominant in shrimp ponds under the same conditions that favor them in freshwater fishponds. These factors include a high phosphorus concentration and a high pH (above 8.3) resulting in low carbon dioxide availability.

The salinity of water in shrimp ponds depends upon the salinity of the water supply and weather conditions. Ponds have traditionally been constructed along estuaries. Salinity decreases with distance upstream from estuaries. During the rainy season, salinity may decrease drastically in estuaries. The relationship between rainfall and salinity at a shrimp farm in Ecuador where the water source is an estuary is illustrated in Figure 1. This figure shows that salinity decreased

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rapidly following the large amount of rainfall in January and remained low until early in the dry season. Of course, salinity did not decline until there had been enough rainfall for freshwater inflow from the drainage basin to dilute the brackishwater in the estuary and ponds. Thus, average monthly salinity was high in January even though the rainfall total was high in the month. In some parts of the world, ponds are filled directly from the sea with water of 30 to 35 ppt salinity, and the salinity may increase to 40 to 50 ppt in the dry season unless water exchange is applied.

In shrimp ponds with clear water, mats of algae known as "lab-lab" may develop on pond bottoms. "Lab-lab" communities often contain blue-green algae, but the occurrence of benthic blue-green algae apparently does not depend upon low salinity. In fact, high salinity water (35 to 45 ppt) often favors communities of filamentous benthic algae ("lab-lab") over phytoplankton and the "lab-lab" in high salinity water is sometimes dominated by blue-green algae.

Shrimp are provided commercial, pelleted feed, but shrimp also graze on detritus on the pond bottom. They ingest considerable amounts of phytoplankton that has settled to the bottom and they feed on benthic algae. As illustrated in Figure 2, shrimp may ingest blue-green algae cells that contain odorous compounds, or they may absorb these compounds directly from the water (*7*).

## Off-flavor in Shrimp

Shrimp processors seldom indicate that off-flavor is a major problem, but most have encountered off-flavor shrimp. Shrimp may be sold as a "heads-on" product or the heads may be removed and only the "tails" sold. Lipids are concentrated in the head of shrimp, and the "tail" muscle is lean. Most producers and processors indicate that off-flavor is more likely to be a problem with "head-on" shrimp than with "tails". This seems logical as heads contain more lipid than tails and geosmin and other odorous compounds tend to concentrate in lipid (*5, 7, 10*). There is a large market in Europe and Japan for "heads-on" shrimp, and the consumers of these shrimp often eat the heads. Nevertheless, Lovell and Broce (*1*) found geosmin concentrations of 78 μg/kg in tail muscle of shrimp. This concentration is greater than the concentration normally found in pond-cultured channel catfish determined to be off-flavor. This finding suggests that off-flavor in shrimp tails also can be of concern.

Processing plants for channel catfish in the United States consider off-flavor a major problem. All purchases of fish from producers require fish to pass a "taste-test" to confirm that the fish are not off-flavor. Shrimp processors do not resort to "taste-tests" as a routine precaution before purchasing shrimp. This

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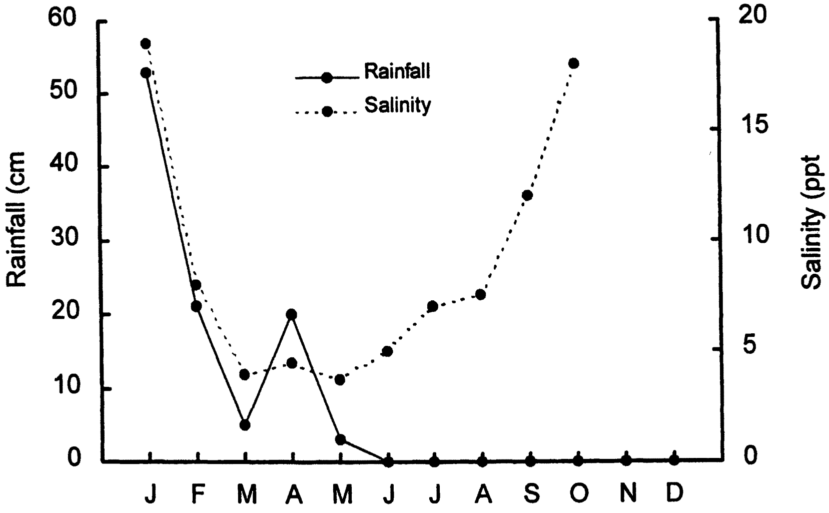

*Figure 1. Relationship between rainfall and salinity in parts per thousand (ppt) in waters of shrimp ponds near Guayaquil, Ecuador. (Reproduced with permission from reference 9. Copyright 1998 Claude E. Boyd.)*

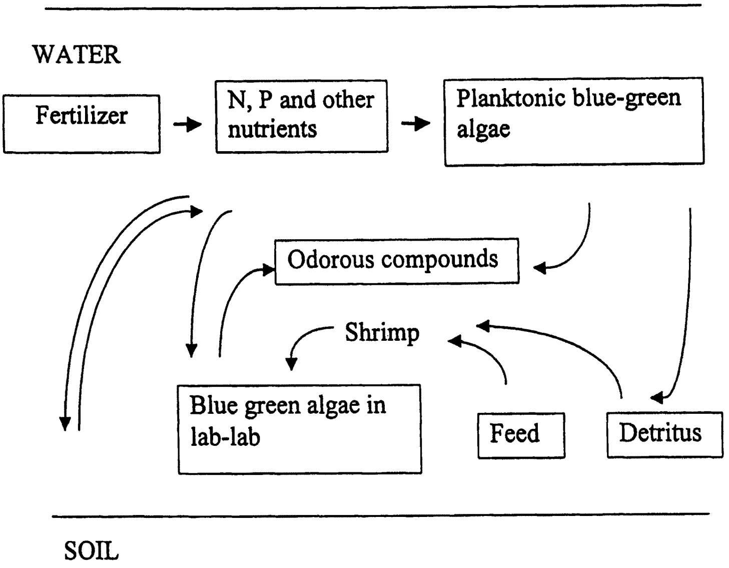
WATER
Fertilizer
N, P and other nutrients
Planktonic blue-green algae
Odorous compounds
Shrimp
Blue green algae in lab-lab
Feed
Detritus
SOIL

*Figure 2. The food web in a shrimp pond showing how shrimp may ingest blue-green algae or detritus containing odorous compounds or how shrimp absorb these compounds from the water.*

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difference in procedure seems to confirm that off-flavor is not as common in shrimp as in channel catfish and other species of fish.

Although off-flavor is not a general concern to shrimp producers and processors, it can occasionally be a very serious problem. In Colombia, a shrimp farm takes water from a small, constricted estuary where salinity may fall below 10 ppt in the rainy season. This farm had operated for several years without encountering problems related to off-flavor shrimp. In the mid 1990's, a project to enhance rice production was initiated on the drainage basin of the estuary. More rice was cultivated and more fertilizer was applied. This resulted in higher inputs of nitrogen and phosphorus to the estuary, and in fall 1993, a dense blue-green algae bloom developed in the estuary. Water from the estuary was used as a water supply for the shrimp farm, and ponds developed dense blooms of blue-green algae. Soon afterwards, shrimp were noted to have a distinct off-flavor and were unacceptable in some markets. There was a freshwater stream nearby, and it was found that the off-flavor in shrimp could be purged by flushing freshwater through a pond for several days before harvest. This practice also has been effective in purging off-flavor from fish (*5, 7*). A more suitable solution to the off-flavor problem was effected by diverting much of the runoff from the rice production area away from the estuary to reduce nutrient input and by recirculating much of the farm water through a nearby mangrove area to effect nutrient removal.

Periodic problems of off-flavor in shrimp may occur during periods of heavy rainfall that cause low salinity in pond waters for several weeks and blue-green algae blooms develop. This phenomenon has been observed in Ecuador, Mexico, Thailand, and other countries. For example, in 1997 and 1998, there was a strong El Niño event in Ecuador and salinity dropped to near zero in many areas following extremely heavy rainfall. There was an increase in complaints about off-flavor shrimp, and producers tried many treatments for reducing blue-green algae blooms without much effect. The only treatment that provided benefit was to increase the water exchange rate to flush blue-green algae and off-flavor compounds from ponds.

Of course, not all problems with shrimp off-flavor are related to low salinity water. Where nutrient inputs to ponds are high, blue-green algae blooms occasionally are observed at moderate to high salinity. There are some farms in Mexico where blue-green algae blooms and associated off-flavor problems have occurred at salinities of 25 to 35 ppt. Off-flavor also has been observed in ponds with salinities of 35 to 45 ppt where dense lab-lab infestations developed. Nevertheless, the occurrence of blue-green algae infestations and off-flavor is thought to be more common in ponds with salinity below 10 ppt than in ponds with greater salinity.

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## Other Problems with Blue-green Algae

In addition to off-flavor, abnormal coloration of harvested shrimp has been associated with blue-green algae. In ponds with high abundance of lab-lab, pigment from blue-green algae apparently can accumulate in the hepatopancreas and in the lipids of the head and impart a yellowish or greenish color to shrimp heads. The color seems to be intensified when the shrimp are cooked, and heads may become noticeably green.

Fouled-gills are a common problem in shrimp, and in some ponds shrimp may have dark blotches beneath the gill covers as the result of trapped soil particles. Filamentous blue-green algae also may become trapped under gill covers giving shrimp heads a distinct green appearance. This coloration is undesirable in shrimp for the "heads-on" market.

Some producers also feel that blue-green algae can be toxic to small shrimp. However, there is incomplete documentation of the validity of this opinion.

Dense blue-green algae blooms can cause low dissolved oxygen concentrations in shrimp ponds during the night just as they do in fishponds (*7*). Low dissolved oxygen concentration is particularly troublesome in semi-intensive shrimp culture in Central and South America because there usually is no way to apply mechanical aeration to ponds which often are as large as 10- to 20-ha in area. The traditional method for preventing dissolved oxygen stress was to use high rates of water exchange to flush offending blooms from ponds. In recent years, a number of serious viral diseases of shrimp have occurred. Water exchange increases the risk of disease because viral particles and their vectors can enter farms via the water supply which may be contaminated with pond effluent from neighboring farms that have been infested with viral diseases. Thus, dense blue-green algae blooms are more undesirable than in the past, for high rates of water exchange are not prudent.

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## Inland Shrimp Farming

In recent years, there has been considerable development of shrimp farms in inland areas of Thailand. Ponds are constructed in freshwater areas, and brine solution from coastal saltwater evaporation ponds is mixed with freshwater to provide salinities of 2 to 5 ppt and allow marine shrimp culture (*11*). It is estimated that 30 to 40% of shrimp production in Thailand currently is derived from inland ponds (*12*). Because of the success of inland shrimp farming in Thailand, the practice has spread to Brazil (*13*), Ecuador (*14*), the United States (*15, 16*), and other nations. Outside of Thailand, the source of water for shrimp culture often is saline water from wells rather than brine solution, but salinity usually is less than 10 ppt in culture ponds.

Inland shrimp farming relies on intensive culture techniques with large nutrient inputs from feed. Salinity often falls to 2 or 3 ppt before shrimp are harvested. In addition, water exchange is not used and ponds are aerated heavily. The culture system is essentially the same one used for intensive production of freshwater fish, and as in freshwater fish culture, blue-green algal blooms frequently develop. It is expected that off-flavor will be a much more serious problem in inland shrimp culture than in traditional, coastal shrimp farming.

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## Blue-green Algae Control

By using water exchange rates of 10 to 20% of pond volume per day, dense blooms of blue-green algae usually can be flushed from coastal shrimp ponds. Aside from this technique, there has been little success in controlling blue-green algae through manipulation of the nitrogen to phosphorus ratio in pond water, application of liming materials, organic matter, and trace elements, additions of probiotics, and use of numerous other amendments. Copper sulfate and other algicides, which have been of some success in combating off-flavor in fish culture (*7*), have seldom been used in shrimp aquaculture to control off-flavor.

In inland shrimp farming in Thailand, farms usually are arranged for water reuse through a reservoir that is 20 to 40% of culture pond area (Figure 3). According to Dr. Chalor Limsuwan of Kasetsart University, Bangkok, Thailand, when off-flavor is observed in shrimp near harvest, heavy water exchange between the pond and the reservoir has been an effective treatment (*12*). Copper sulfate treatment at one-one hundredth of the total alkalinity, as used in channel catfish culture for off-flavor control (*7*), possibly could be a useful method in inland shrimp culture.

## Conclusions

Off-flavor can occur in pond culture of shrimp when shrimp ingest or absorb geosmin and other odorous compounds from blue-green algae and actinomycetes just as happens in freshwater, pond-fish culture. However, the occurrence of off-flavor in shrimp culture is less common than in freshwater fish culture. The reason for this difference is related to a low abundance of blue-green algae in most shrimp ponds because they have high salinity water. The abundance of blue-green algae and the occurrence of off-flavor in shrimp

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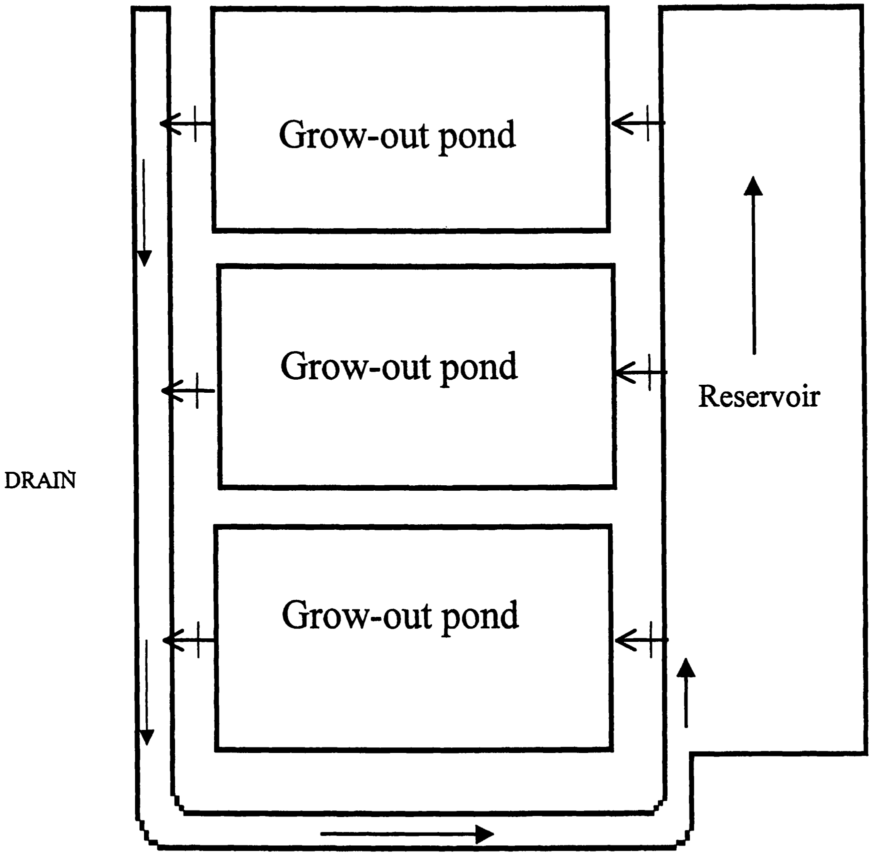
Grow-out pond
Grow-out pond
Grow-out pond
Reservoir
DRAIN

*Figure 3. Illustration of a small, inland shrimp farm arranged for water re-use. Water can be saved when ponds are drained and water can be exchanged between any pond and the reservoir when desired.*

tend to increase in ponds with salinities below 10 ppt. Off-flavor is expected to be a more serious problem in inland shrimp farming in low-salinity water than it is in coastal shrimp ponds.

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## Chapter 5

# Off-Flavor Problems and a Potential Solution within the U.K. Trout Industry

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## Abstract

The cause of earthy-musty taints on UK rainbow trout (*Oncorhyncus mykiss*) farms was investigated. Microwave distillation followed by solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) was used to quantify fish taint levels. Geosmin was found to be the main compound associated with the seasonal occurrence of earthy-taints in UK farmed trout (both in southern and northern regions). In most fish, 2-MIB was recorded close to or below detectable levels suggesting it had a negligible influence on earthy taint episodes. In a river-fed northern UK farm (soft-water), the seasonal build up of epilithic GSM-producing *Oscillatoria* sp. was attributed to the cause of tainting episodes. Whether or not this species is the cause of taints in the more alkaline waters of southern UK farms is not known. Comparison between organoleptic scores and chemical analysis by SPME-GC-MS estimated the threshold of geosmin in trout at circa 1.5 µg/kg. UV/$TiO_2$ photocatalyis was shown to destroy 2-MIB and GSM and may provide a potential solution for removing odorous taints from water supplies. From an initial taint concentration of 2 µg/L, 50 % of 2-MIB and GSM was destroyed in 2.5 and 15 min, respectively.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

# Introduction

The UK farmed trout industry is small compared to UK marine aquaculture where salmon (*Salmo salar*) production is ten fold that of rainbow trout, *Oncorhyncus mykiss*. This may in part explain the lack of information in the literature on off-flavor problems in UK trout. As far as the authors are aware the only research conducted on earthy taint in UK waters is on wild salmon from Irish rivers (*1*). Our current understanding of the causes and potential solutions for dealing with off-flavors in farmed fish is primarily based on the channel catfish, *Ictalurus punctatus,* industry of the USA. Much has been written on this subject over the past three decades and has recently been extensively reviewed (*2*). A key finding of a recent UK survey revealed that 20% of farms reported incidences of earthy taints on a seasonal basis. In these cases, 90 % of them used river water as their main supply to the farm, however production systems varied and tainting arose in raceways, earth ponds, and combination systems to similar extents.

Two lipophilic compounds, geosmin (GSM) and 2-methylisoborneol (2-MIB), have been attributed to the majority of cases of earthy-musty odors in farmed freshwater fish (*2*). GSM and 2-MIB have been shown to be produced by two groups of microbes, namely the actinomycetes and cyanobacteria (*3*), with the latter group being credited as the causative organism in most reported cases of earthy taints in fish (*2*). These compounds have been classed as harmless at environmental levels (*4,5*) but can cause marketability problems due to their rapid bioaccumulation into lipid rich tissues of fish (*6,7,8*). Retaining tainted stock that has been rejected by processors can cause significant economic losses for fish farmers (*9,10*).

The are two basic strategies to prevent the tainting of marketable fish. Firstly, by controlling the growth of cyanobacteria in ponds, the capacity for taint production may be reduced or, secondly, the tainting compounds may be removed. The first option generally involves the application of copper-based algicides but other novel compounds have been investigated (11). The second option relies on the fact that fish can purge themselves of taints (*8,12,13*). However, a water treatment system must be employed to supply taint free water to purge ponds unless a guaranteed taint free source is available, e.g., bore hole water.

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Many methods have been investigated for the removal of taints from water, specifically, the use of granular activated carbon (GAC), powder activated carbon (PAC), ozonation, peroxone and bio-filtration (*14,15,16,17*). The success of such treatments to provide taint free water for purge tanks has been limited. This is in part due to the effectiveness of these technologies in removing sufficient quantities of taints rapidly to maintain concentrations below the level that can cause odor problems. UV/Titanium dioxide ($TiO_2$) photocatalysis has been shown to destroy other cyanobacteria metabolites (*18,19,20*) as well as a wide range of harmful organic compounds. $TiO_2$ acts a semiconductor when illuminated by UV and generates hydroxyl free radicals as well as superoxide anions which, in water, rapidly convert to hydrogen peroxide providing further oxidative potential (*21*). This paper will investigate the cause of earthy taints in UK farmed trout as well as evaluate the potential of UV/$TiO_2$ to destroy GSM and 2-MIB.

## Materials and Methods

### Algal Sampling and Isolation

A visual record of the development of the biofilm (including epilithic cyanobacteria) on the inside of concrete raceways was achieved by taking a scrape sample from the inside of the raceway wall using a 30 mL sterile plastic universal. Sub-samples from these scrapes were then prepared on microscope slides and photographed. Further sub-samples from raceway algal scrapes were cultured on BG-11 plates (1.5% agar) to which cycloheximide (5 µg/mL) had been added to inhibit the growth of eukaryotic green algae. Several replatings of isolates were necessary to obtain isolated colonies of cyanobacteria. Using aseptic techniques, isolates were transferred into 25 mL liquid BG-11 media in 50 mL conical flasks then subsequently transferred to 100 mL media in 250 mL conical flasks. Plates and culture flasks were incubated at 25 °C under continuous illumination. Culture isolates and biofilm samples were analyzed by SPME-GC-MS for taint compounds according to the method of Watson and co-workers for water samples (*22*). For SPME sampling, media and cells were separated by filtration, 25 mL of media in 40 mL vials (containing 6 g NaCl, and a PFTE mixer bar) were heated to 65 °C and analyzed for taints. Cells were transferred to 40 mL vials and frozen at -20 °C to promote lysis. Once defrosted, the volume of liquid in the vials were broght to 25 mL with milli Q™ water.

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## Taint Sampling of Trout

The trout were sexed, weighed, gutted and placed individually into freezer bags. Individual bags were then put into a second freezer bag along with the completed sample identification card. Air was removed from the bag before sealing and the samples were frozen at -20 °C until required. Fish samples were analyzed for the earthy taint compounds GSM and 2-MIB using microwave distillation followed by solid phase microextraction (SPME) and analyzed using gas chromatography-mass spectrometry (GC-MS) according to the recently published method of Grimm and co-workers (*23*) with the following modifications. An analogous microwave set-up to that of Grimm *et al.* was constructed. Sub-samples (20 g± 0.5) of trout fillets were taken perpendicular to the dorsal fin (including the skin) and microwaved in a sealed vessel for 3 min at 440 w. The fiber (65 μm polydimethylsiloxane-divinylbenzene) was exposed to the headspace for 15 min. Thereafter it was retracted and injected onto a 30 m x 0.25 μm x 0.25 mm ZB-5 column (Phenomenex, UK). Daily calibrations were performed using 2-MIB and GSM analytical standards (Supelco, UK) which were diluted with milli Q ™ water to achieve standards over the range of 0.1 to 5 μg/L.

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## Comparison of Organoleptic Scores with Chemical Analysis

A major UK fish processor using 4 panelists who were trained in-house conducted the taste testing. Half a fillet of fish was cooked in a microwave assessed and classed on a four-point scale. The earthy flavor scale was as follows; no taint, very slight taint, slightly tainted and tainted. The other halves of the fillets were frozen prior to chemical analysis by SPME-GC-MS.

## UV/TiO$_2$ Photocatalysis

UV/TiO$_2$ photocatalysis experiments were conducted using 10 mL of aqueous solutions of either GSM or 2-MIB (2 μg/L). Samples were illuminated in the presence of air and TiO$_2$ catalyst (P-25 Degussa, Germany; 1% w/v) using a xenon UV lamp (480 W UVA Spot 400 lamp, Uvalight Technology Ltd; spectral output 330–450 nm). Reactions were carried out in glass universal bottles with constant stirring. Samples were taken at timed intervals and centrifuged (13000 x g) to remove the catalyst prior to the determination of taint levels using the SPME-GC-MS method.

### Statistical Analysis

All data are presented as means ± standard error of the mean (SEM) and n refers to the number of samples. The differences between means were tested using a one way anova followed by a Tukey test. The significance of any differences between mean values was determined at the 95% level of confidence ($P<0.05$), unless otherwise stated. Statistical analysis was performed using the computer statistical package Graphpad Instat (Graphpad Software Inc, USA).

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## Results and Discussion

### The Tainting Compound

Fish from seven northern UK farms were analyzed using SPME-GC-MS and compared to organoleptic taste scores for earthy-musty taints (Figure 1). The differences between the taint score groups (non tainted, very slightly tainted, slight tainted and tainted) were only significantly between those classified as non tainted and tainted (Anova with Tukey test, $p<0.01$). Non tainted fish and tainted fish had a GSM concentration of 1.04±0.11 (range, 0.54-1.51) and 2.69±0.41 μg/kg (1.78-4.02), respectively. The data for the intermediate groups (very slightly tainted and slightly tainted) was variable and not significantly different from each other or any other group. This may be a reflection of the difficulties that the taste panel had in classifying such fish into intermediate groups. Further work is planned to refine our estimate of the GSM sensory threshold in trout. This will involve a larger sample size with a revised taint scale that has only one intermediate class, namely, slightly tainted. However, it can be still estimated from figure 1 that GSM sensory threshold is circa 1.5 μg/kg.

Levels of 2-MIB in the northern UK fish were less than or close to detectable limits suggesting that GSM was the primary cause of earthy taints in this region. Our provisional estimate of 1.5 μg/kg for the tainting threshold of GSM in rainbow trout is lower than the previous estimate of between 6.0 and 10.0 μg/kg for rainbow trout (*13, 26*) and 8.0 μg/kg for catfish, *Ictalurus punctatus* (*25*). These differences may reflect advances in analytical techniques and/or variation

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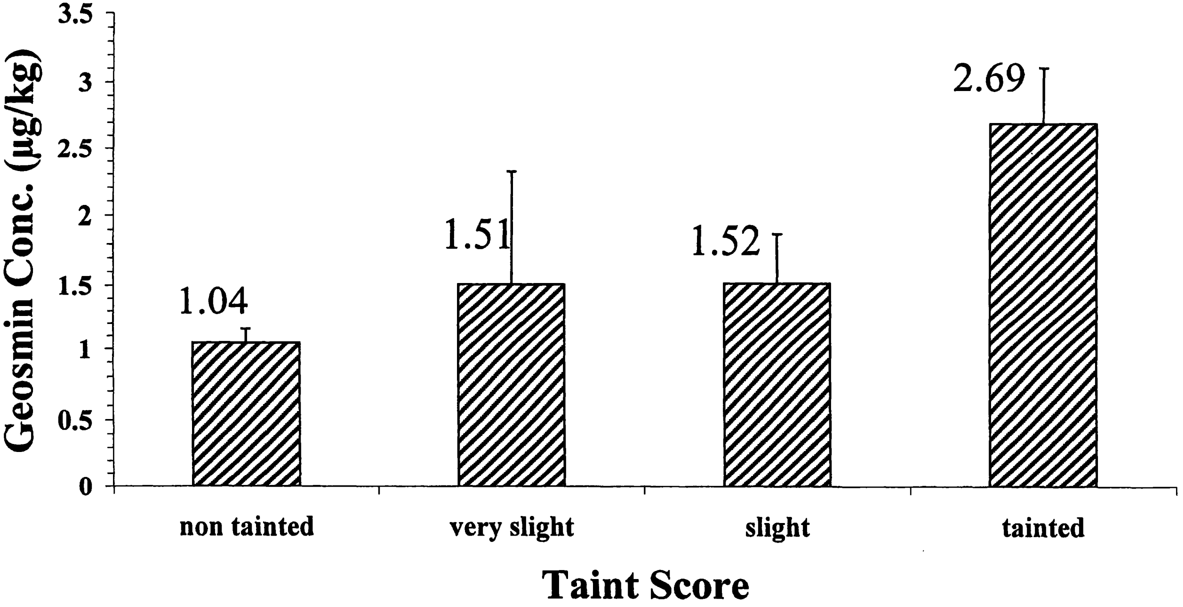

*Figure 1. Organoleptic scores for geosmin against chemical analysis by SPME-GC-MS (means ±SE, n =18).*

in taste panel protocols. However, our threshold estimate is close to that recently reported in the tilapia, *Oreochromis niloticus* (1.5-2.6 μg/kg) (*24*).

GSM and 2-MIB levels based on pooled samples from both northern and southern UK farms collected over a year sampling period are shown in Table I. From this table it can be seen that 2-MIB levels in trout are low in both regions with 82 % and 76 % of southern and northern fish, respectively, having levels at or below 0.1 μg/kg. No cases of 2-MIB were recorded above 0.4 μg/kg in either region. Based on the 2-MIB threshold estimate of 0.6 μg/kg for trout (*26*) and 0.7 μg/kg for catfish (*27*), it is unlikely that 2-MIB is the cause of taint problems in UK fish. However, Table I shows much higher reported levels for GSM in both regions. Figure 2 typifies this pattern and shows a seasonal accumulation of GSM in fish on a northern UK farm. GSM in trout peaked over the warmer summer months and showed a steady decline in late autumn and winter months. On the farm investigated, 2-MIB levels remained low throughout the whole season and were regarded as having little influence on the tainting of fish.

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*Table I. SPME-GC-MS analysis of taint levels in trout from southern (n=100) and northern (n=127) regions of the UK.*

| ***2-MIB*** | ***UK Region*** | | ***GSM*** | ***UK Region*** | |
|---|---|---|---|---|---|
| ***concentration range (μg/kg)*** | ***South (%)*** | ***North (%)*** | ***Concentration range (μg/kg)*** | ***South (%)*** | ***North (%)*** |
| <0.1 | 82.0 | 75.6 | <0.3 | 15.0 | 6.3 |
| >0.1<0.3 | 16.0 | 20.5 | >0.3<1.5 | 65.0 | 67.5 |
| >0.3<0.4 | 2.0 | 3.9 | >1.5<3.0 | 11.0 | 23.0 |
| | | | >3.0<7.2 | 9.0 | 3.2 |

The seasonal nature of tainting episodes on fish farms have been well documented, especially when water temperatures increase in the range 15-25 °C (*2*). In Table I it can be seen that 85 % and 70.8% of southern and northern, respectively, were at or below 1.5 μg/kg. Based on our estimate of the taint threshold of GSM (~1.5 μg/kg) in trout, these fish would be chemically classified as non-tainted. Fish GSM levels in the range >1.5<3.0 μg/kg, were 11 % and 23 % for southern and northern, respectively. For values greater than 3.0 μg/kg, 9.0 % and 3.2 % were found in southern and northern fish, respectively. The maximum recorded value for GSM measured in trout was 7.2 μg/kg.

## The Causative Organism

No apparent turbidity due to planktonic cyanobacteria was observed within farms. In contrast, microscopic analysis of tank wall scrapes taken on northern

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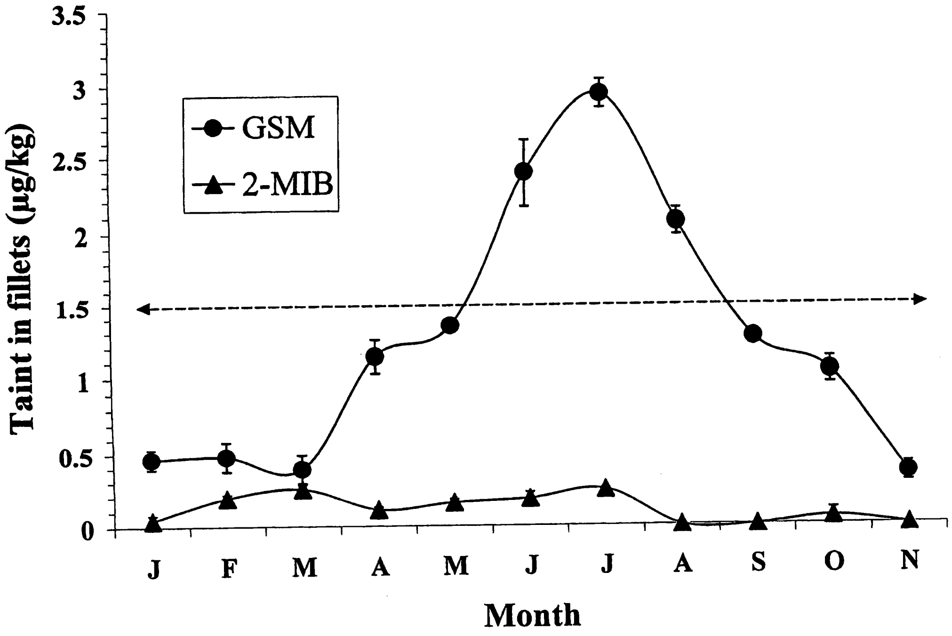

*Figure 2. SPME-GC-MS analysis of taint levels in rainbow trout on northern UK farm (means ±SE, n=3). The dashed arrowed line represents our estimate for the geosmin sensory threshold (1.5 µg/kg) in trout.*

UK trout farms have shown gradual accumulation of cyanobacteria from early March to peak abundance and dominance of the raceway biofilm in July. Thereafter, there was a gradual decline in the amount of cyanobacteria toward October. From November to February, photographic records of the microscopic analysis show the presence of cyanobacteria to be rare or absent. Field observations have also confirmed high abundance of epilithic cyanobacteria attached to rocks upstream of the farms studied. This seasonal pattern of cyanobacterial accumulation matched the seasonal rise in GSM in trout within one of the farms monitored (Figure 2). As some cyanobacteria are known to be earthy taint producers (*3*), the correlation between the increasing presence of this microbe and the increasing incidents of tainted fish suggested a link.

The microbe of interest was isolated using standard microbial techniques and is suspected of being a GSM producer. The cyanobacteria was identified as being a member of the genus *Oscillatoria* (Figure 3) and no other species of cyanobacteria were found in tank scrapes and during subsequent isolation attempts on these samples. However, this does not necessarily mean that this *Oscillatoria* species is the causative organism for all reported cases of earthy taints in UK fish, as other species in other UK regions may also produce GSM. The majority of northern UK trout farms can be classed as river-fed or loch-based farms using soft-water; in contrast, southern UK farms can typically be classified as hard-water river-fed systems. Variations in water hardness and pH can affect the species composition of microbes and, thus, the species of taint produced. For example, in Mississippi catfish farms, the major tainting compound is 2-MIB and this has been attributed to *Oscillatoria* spp. (*28*), whereas, in the more acidic waters of Alabama, GSM is the main problem which has been linked to the presence of *Anabaena* spp. (*29, 30*). As GSM appears to be the main UK tainting compound, this will influence the focus of UK research towards evaluating technologies to destroy or remove GSM from water and investigating the kinetics of uptake and depuration rates of GSM in trout.

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## UV/$TiO_2$ Photocatalyis

From an initial starting taint concentration of 2 μg/L, it can be seen in Figure 4 that 50 % of 2-MIB and GSM is destroyed in 2.5 and 15 min, respectively. Although we have clearly shown that UV/$TiO_2$ photocatalysis can rapidly destroy odorous earthy taints, the reaction has not yet been fully optimized. It has also been shown that the addition of hydrogen peroxide to the

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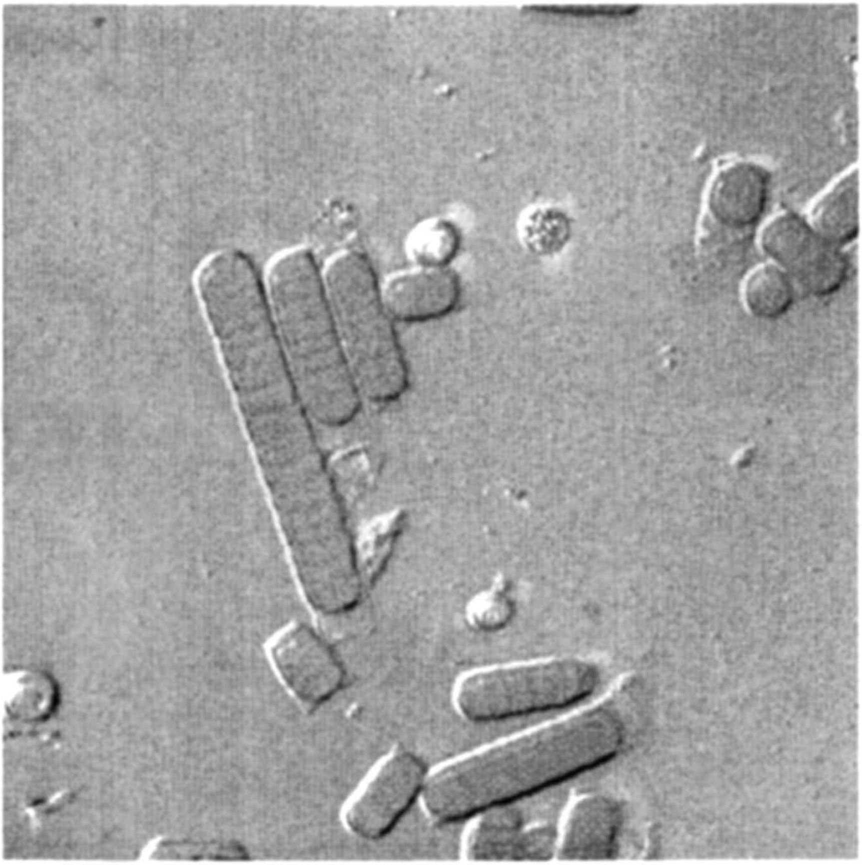

*Figure 3. Micrograph of a geosmin-producing isolate (Oscillatoria sp.) from a northern UK trout farm (x400 mag).*

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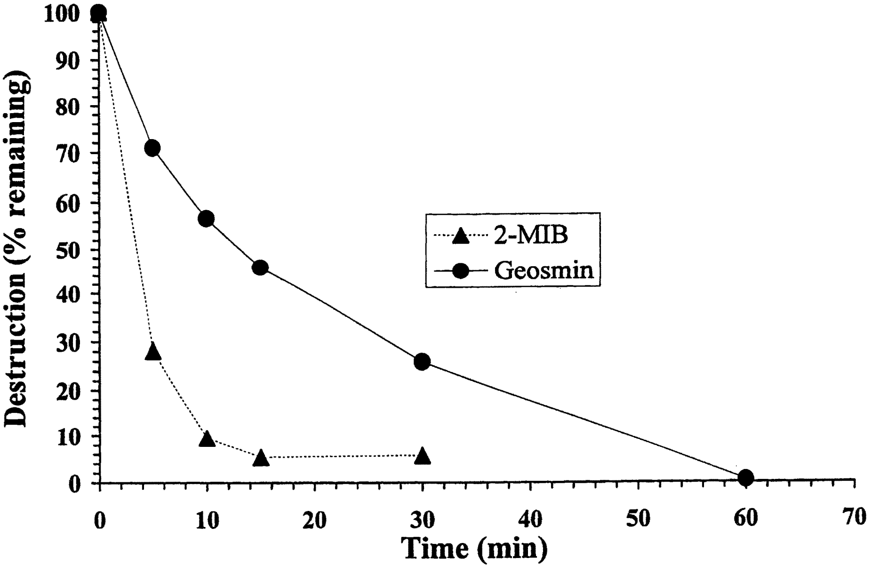

*Figure 4. Destruction curves for GSM and 2-MIB using UV/$TiO_2$ photocatalysis. Initial taint concentration was 2 μg/L. The amount of catalyst used was 1 % $TiO_2$ (w/v) operated at room temperature.*

photocatalytic system can enhance rates of destruction of non-desirable compounds (*19*). The effects of temperature, pH and organic loadings on reaction rates should be studied to judge the potential of this novel water treatment system, as well as the economics of its application to aquaculture. In addition to having a greater oxidizing potential compared to other water treatments (*19*), $TiO_2$ has many other advantages. The $TiO_2$ catalyst is cheap, requires no additional chemicals and $TiO_2$ has "self cleaning" properties. Compounds are destroyed unlike in the case of GAC where they are adsorbed to the surface of the carbon. This also means there are no spent filter disposal costs thus overall operational costs may be more economical.

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## Conclusions and Summary

We can conclude from our data that in those fish sampled, geosmin was found to be the main compound associated with the seasonal occurrence of earthy taints in UK farmed trout (both in southern and northern regions). In most fish, 2-MIB was recorded in fish close to or below detectable levels suggesting it had a negligible influence on earthy taint episodes. In a river-fed northern UK farm (soft-water), the seasonal build up of an epilithic GSM-producing *Oscillatoria* sp. was linked to the cause of tainting episodes. Whether or not this species is the cause of taints in the more alkaline waters of southern UK farms is not currently known. Comparison between organoleptic scores and chemical analysis identified the threshold of geosmin in trout at around 1.5 µg/kg. UV/$TiO_2$ photocatalysis can destroy 2-MIB and GSM and may provide a potential solution to removing odorous taints from water supplies.

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## Chapter 6

# Natural and Waterborne Petroleum Hydrocarbons in Marine Sentinel Blue Mussels

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Some chemicals produced by algae are water soluble, volatile, or both. They may be offensive in certain concentrations and attractive at others (e.g. dimethyl sulfide in cooking clams). Quality grading, safety, and regulatory inspections are factors often in use before consumers ever see foods. The frequent use of petroleum hydrocarbons in our world has led to reports of environmental contamination, and mussels were selected as very convenient bioconcentrators for helping to identify organic pollutants in marine waters. Low-boiling petroleum fractions have alkane profiles similar to the C10-C24 *n*-alkanes present as a natural background from algal hydrocarbons and are almost always present in ocean water. This review suggests that the ratio of two common isoprenoid hydrocarbons, pristane and phytane, may be important in distinguishing the profiles of biogenic and abiogenic hydrocarbons. General problems with mussels, used as sentinels for examining this range of hydrocarbons, are briefly reviewed.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

## Natural "Chemicals" in the Oceans

The oceans are sources of an almost infinite number of organic compounds, many of high-level structural complexity. Most of these compounds are accumulated by or "locked up" in living organisms, some impart characteristic flavors to aquatic food products (*1*). Other compounds are quite simply chemical and of no public interest unless apparent in some active and usually irritating form. Two examples of simple compounds will illustrate how these natural processes function.

Bromophenols became familiar to New England and Nova Scotia fishermen in the form of the algal product 2,4-bromophenol during the harvesting of a deepwater bivalve, the ocean (or mahogany) quahaug (*Arctica islandica*). Quite suddenly landings with an "iodine" flavor developed and caused a large drop in consumer demand. These industries have suffered on more than one occasion with 2,4-dibromophenol identified as the culprit. 2,4-Dibromophenol probably originated in an unnoticed phytoplankton bloom, and, after the cells died and drifted to the sea floor, they would have resisted common bacterial degradation due to the presence of 2,4-dibromophenol (*2*). The remains of the cells may have eventually formed part of the seston, a haze of miscellaneous detritus in a layer very near the sea floor (*3*). This habitat feature conforms to probable sources of the diets of quahaugs. The bromophenols are also currently a problem in Australian fisheries, demonstrating the worldwide impact of some algal product problems; however, their exact origin and biochemical basis often remains obscure (*4*).

Dimethyl- -propiothetin (DMPT), also of phytoplankton origin, can be accumulated in Arctic waters in small, black snail-like organisms *Limacina helicina*. They are eaten by fish to the extent that stomachs full of black "granules" were referred to in the U.K. as "the gunpowder problem". The problem is that DMPT breaks down (Figure 1) to yield dimethyl sulfide (DMS) which is very similar to gasoline in aroma (*5*).

Fortuitously, during the development of northern fisheries in the 1960s, gas-liquid chromatography was introduced thereby facilitating the identification and recognition of this problem (*6, 7*). Table I illustrates the very low levels of DMS required to condemn fish with a hydrocarbon type odor, the result of feeding on *L. helicina* in an unusual influx of Arctic water near Halifax, Nova Scotia. This case illustrates that only trace odors may be required with sensitive consumers and regulatory inspectors to cause shellfish or fish to be rejected.

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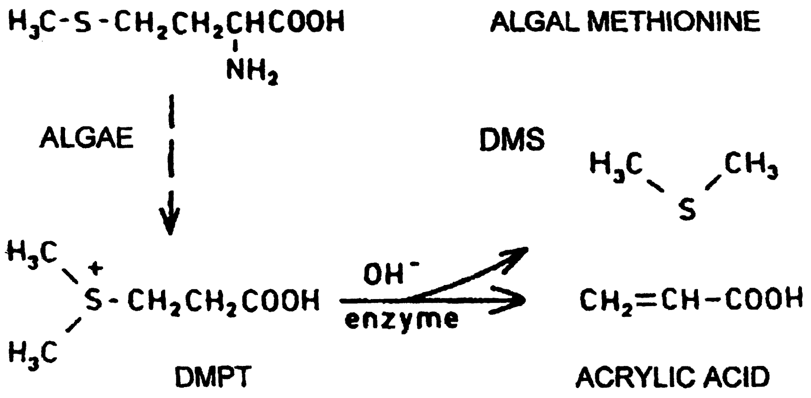

Figure 1. Biosynthesis and decomposition of dimethyl- -propiothetin (DMPT).

Table I. Levels of dimethyl- -propiothetin (DMPT, reported as hydrochloride) and dimethyl sulfide (DMS) in Eastern Passage mackerel (*Scomber scombrus*) rejected by Government inspectors because of a petroleum smell.

| DMPT (mg/g) | | | DMS (mg/g) | | |
|---|---|---|---|---|---|
| Stomach | Tail muscle | Belly flap | Stomach | Tail muscle | Belly flap |
| 0.002 | 0.0001 | 0.0004 | 0.002 | 0.0002 | 0.0002 |
| 0.008 | 0.0002 | 0.0003 | 0.017 | 0.0003 | 0.0002 |
| 0.001 | – | – | 0.001 | -- | – |
| <0.001 | – | – | <0.001 | -- | – |

## The Petroleum Problem

In the modern world, petroleum products and, to a lesser extent, petroleum product spills in marine environments, are a fact of daily life. For example, in the year 2000, the staff of the International Tanker Owners Pollution Federation Limited assisted in eighteen major incidents requiring their expert input for control or cleanup (*8*). Infamous spills have ship names attached to them, such as the

Braer incident off the Shetlands Islands (*9*) or the Exxon Valdez incident in Alaska (*10*). The toxic effects of such spills are heavily influenced by weathering (*11*), and long-term effects are still being evaluated (*12*). Production of petroleum from the floor of the oceans is expanding and our research group has been successful in participating in monitoring operations near petroleum production facilities on the Scotian Shelf off Sable Island, Nova Scotia. The common blue mussel (*Mytilus edulis*) was the indicator species monitored in our studies (*12*, *13*). The subject of hydrocarbon monitoring, focusing primarily on the alkanes produced by planktonic marine algae and present in seawater, separates this work from the world-wide use of mussels for assessing health risks from polycyclic aromatic compounds. The latter were as little as 0.2% of the Scotian Shelf light crude petroleum, so distinguishing between the alkane profile from the seawater and that in the petroleum became our primary objective at that time.

Other related investigations have included early introduction of steam distillation (*15*) and application of this method to hydrocarbons in seawater under ice cover (*16*) and to lobster meat (*17*). Later publications include tests of other potentially useful recovery techniques (*18, 19*) and partitioning of petroleum soluble hydrocarbon into seawater on a continuous basis (*20*) with contaminated seawater produced for experiments and stored in plastic bags (*21*).

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## The Isoprenoid Ratio Factor in Differentiating Biogenic and Abiogenic Hydrocarbons

Coastal waters near most urban areas may be suspected of hydrocarbon contamination (*22, 23, 24, 25*). A particular hydrocarbon contamination case in 1975 included the analysis of hydrocarbons in tissues of lobster (*Homarus americanus*) exposed to diesel oil (*17*). This case showed that alkane hydrocarbons, C16-C21, were present in both control and contaminated lobster in similar proportions. However, relative to phytane, the amount of pristane was remarkably higher in the control lobster meats than in the contaminated meats. At the time, there was little information comparing the contamination of the meats and the natural marine hydrocarbons in natural samples despite the excellent work of M. Blumer, J.W. Farrington, and others (*26*).

The point of interest is that both sources of isoprenoid hydrocarbons are derived from phytol (Figure 2). This component of chlorophyll may be mildly toxic to invertebrates such as copepods that store lipids in oil sacs so they can either oxidize it to phytanic and/or pristanic acids (not shown) or convert it to phytane (2,6, 10,14-tetramethylhexadecane) and pristane (2,6,10,14-tetramethylpentadecane). In the oceans any of these compounds can also be degraded to 4,8,12-trimethyltridecanoic acid which, together with pristanic and phytanic acids, is

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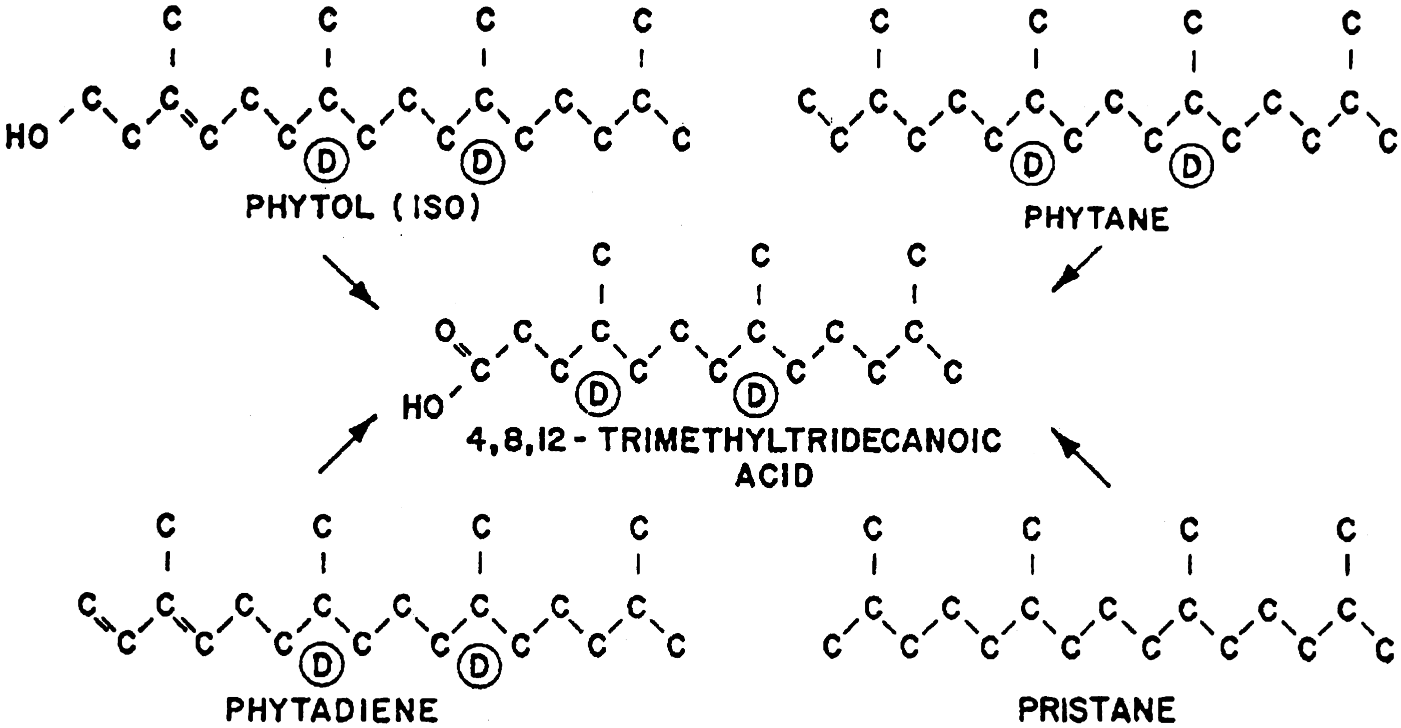
PHYTOL (ISO)
PHYTANE
4,8,12 - TRIMETHYLTRIDECANOIC ACID
PHYTADIENE
PRISTANE
HO
O
C
D

*Figure 2.* *Several possible precursors of 4,8,12-trimethyltridecanoic acid. Note the common sterochemistry of three precursors. No stereochemistry is indicated for pristane.*

found in all fish oils (*27*). The occurrence of pristane applies equally to freshwater fish lipids (*28*). In the oceans, phytadienes have long been recognized as another plausible product of phytol modification, presumably by biochemical action.

The formation of petroleum from decaying organic matter that contains phytol is anaerobic and can lead to the formation of pristane and phytane. However, in some formations such as the Green River Shale, the isoprenoid fatty acids can persist unchanged except for similar shortening of the chain at the original alcohol end of phytol (*29*).

In examining the light petroleum from the Scotian Shelf, it was noted (Figure 3) that the pristane:phytane ratio was approximately 4:1. We have examined hydrocarbons in the mussels and there is far more pristane and, in fact, phytane can sometimes hardly be observed, although sample hydrogenation increases the phytane peak considerably, presumably from phytadienes.

In recent years, examination of mussels, either cultured inshore or from monitoring activity near Sable Island, has produced profiles dominated by pristane with little or no phytane being detected. This observation leads us to believe that the ratio pristane:phytane could be an important indicator of crude petroleum input to the complex alkane profiles recovered from mussels. If the ratio is very low, contamination is likely. More accurate data is needed to fully test this hypothesis. A particularly good reason for assessing the two fully saturated alkane hydrocarbons is their similarity in structure and proximity in gas-liquid chromatography profiles. Almost any recovery and isolation technology will be consistent for proportions of these two hydrocarbons.

Short and Harris (*10*) have discussed the erratic natural production of pristane found in mussels at the site of the Exxon Valdez oil spill. This occurrence suggests that a ratio relative to phytane might also give more consistent interpretation of results than rather simple concentrations presented in numerous tables.

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## Sentinel Animals

### Crabs as Monitoring Animals

Although snow crabs (*Chionoecetes opilio*) are unlikely to be used for the long-term exposure studies practical with mussels, they are found on the Scotian Shelf and elsewhere in the Atlantic Provinces. Snow crabs could potentially be fished regularly near petroleum operations for hydrocarbon analysis, and they are essentially scavengers and/or carnivores. There are two sources for examination, the hepatopancreas (2-3%) and the flesh (ca. 0.7%). The hydrocarbons in the former would reflect long-term storage of exogenous hydrocarbons and in the latter more recent levels of water-soluble hydrocarbons.

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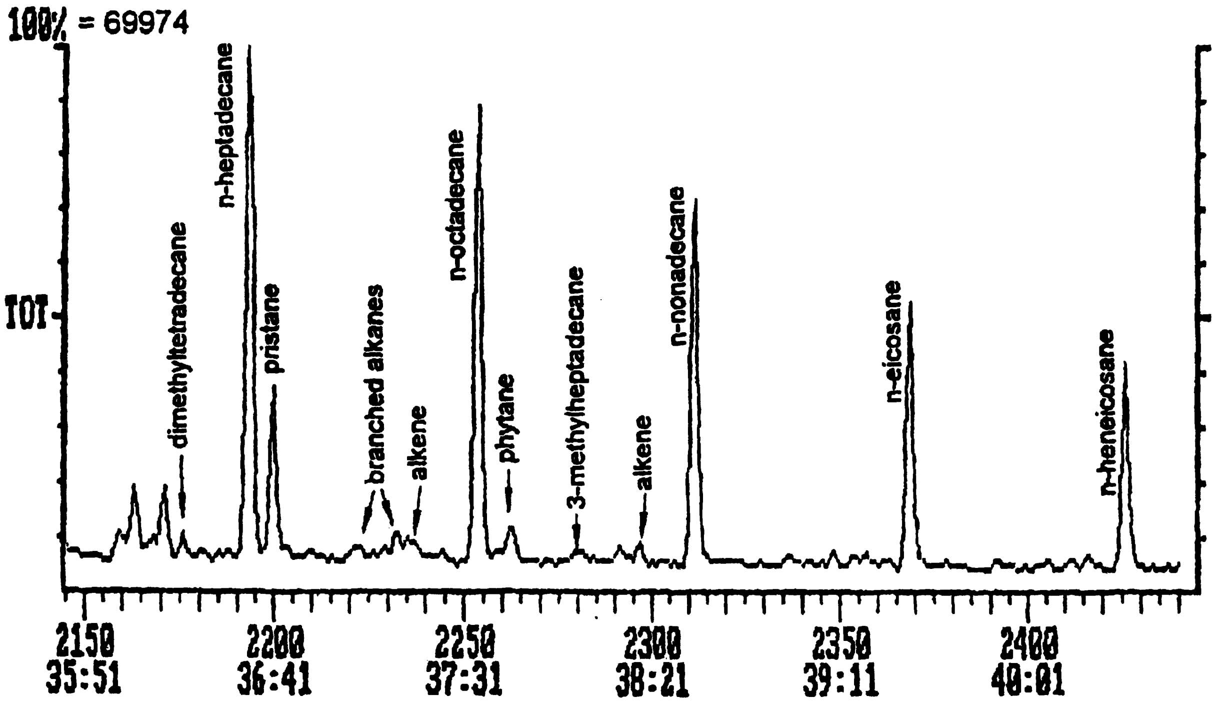

*Figure 3. Partial ion chromatogram of water-accommodated Scotian Shelf light petroleum.*

The gas-chromatographic baselines of analyses of hydrocarbons recovered from snow crabs were free of the biogenic algal hydrocarbons such as the C21 polyunsaturated hydrocarbons that are often characteristic of the mussels (Figure 4). Otherwise, the flesh profile hydrocarbons show the same natural hydrocarbon profiles (C10-C24) in both control snow crabs from another area (Figure 5), and snow crabs from an active petroleum production area. The pristane was a very substantial peak in both crab and mussel lipid hydrocarbon analyses from the latter area of approximately the same time of the year (R.G. Ackman, unpublished results).

## Problems with Mussels as Monitoring Animals

The original decision to use common blue mussels for the "musselwatch" program for the evaluation of pollutants was partially based on their worldwide occurrence. One problem that may not have been apparent at first is the frequent low level of lipid present in mussels, presumably where the living animals store the more lipophilic materials. We have shown that in salmon the hydrocarbons dissolved in sea water are distributed between the intercellular fluids and the adipocytes (*30*), but this distribution is not as clearly defined in mussels with relatively low lipid levels, 1-3% of wet weight being normal for well-fed mussels (*31, 32, 33*). The presence of gonads is also a particularly large source of variation (*31*). However, one recent report states that the water concentration of hydrocarbons is more important than the lipid content of the wet tissue (*33*).

## Problems with Methods

The recovery of mussel lipids can be very simple, for example, by blending mussel in hexane with anhydrous sodium sulphate used for drying (*32*), or can somewhat be more complicated, such as the method of Bligh and Dryer based upon chloroform and methanol (*34*). This latter method was highly regarded and universally popular for forty years, even though it was designed expressly for lipid recovery from lean fish muscle with a high content of phospholipids. Subsequently the method was shown to give inconsistent results for non-fatty-acid materials such as alpha-tocopherol (*35*), and more recently to be unsuitable for high-fat fish muscle (*36*). Similarly, a Soxhlet extraction of fish muscle with hexane/acetone gave different results for PCB congeners than did chloroform/methanol (*37*).

It is opportune to point out that in Europe there is a relatively unknown environmental group called "QUASIMEME" (*38*) that has been examining this problem in some detail (*39, 40, 41, 42*). Smedes (*42*) discussed in detail the use of non-chlorinated solvents. Although the solvent choices are influenced recently

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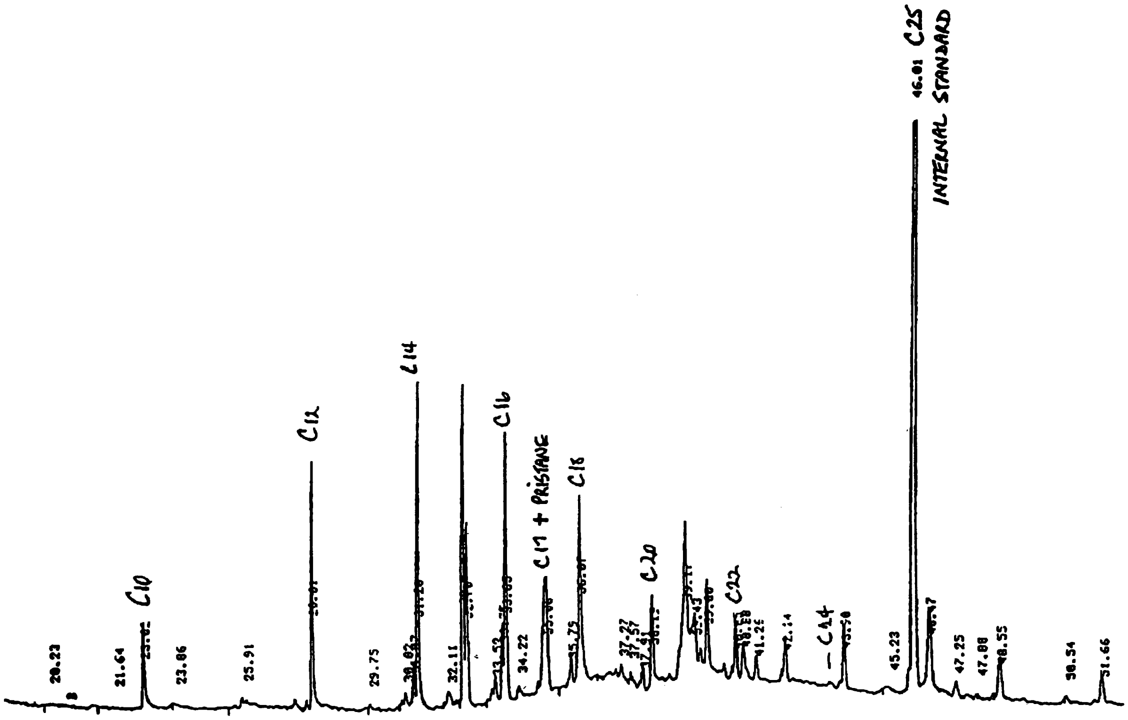

*Figure 4. Partial gas-chromatogram of hydrocarbons recovered from retail aquaculture mussels contemporaneous with the snow crab of Figure 5. Note cluster of C21 hydrocarbons peaks between the n-alkanes C20 and C22.*

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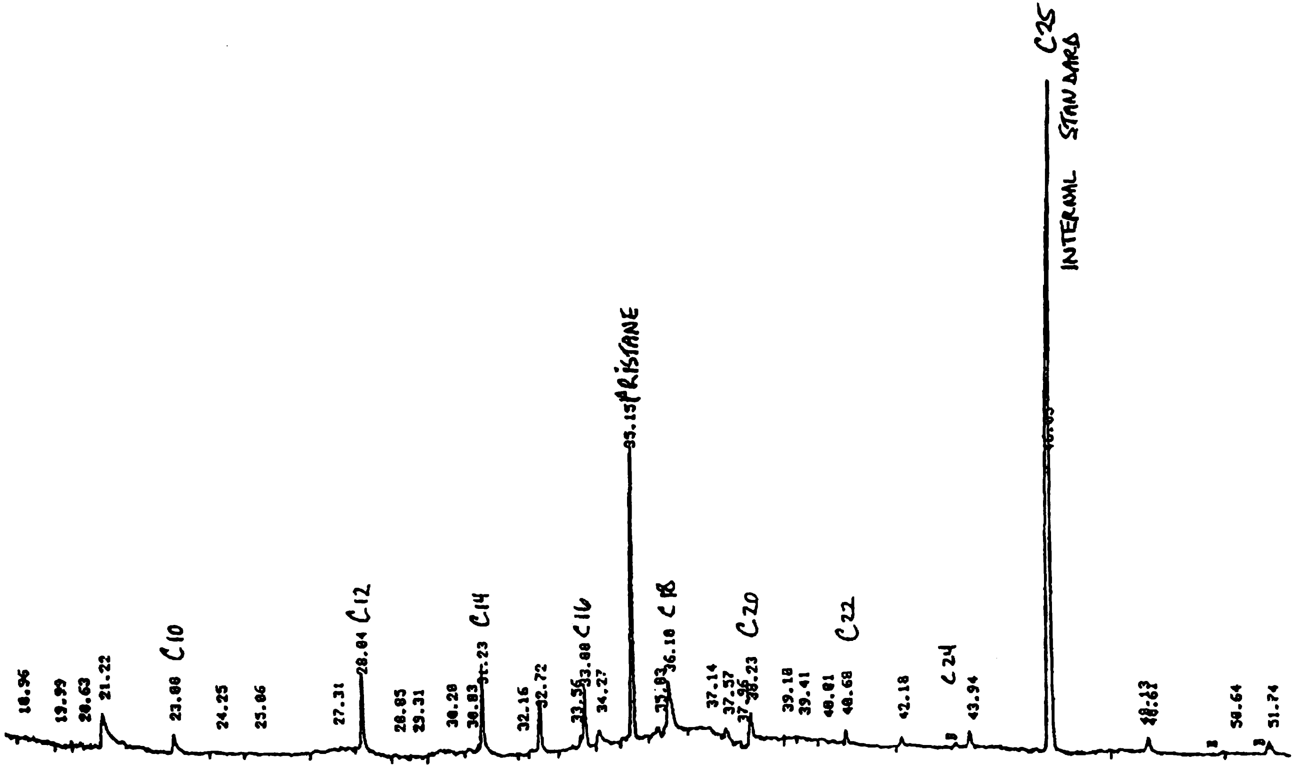

*Figure 5. Partial gas-chromatogram of hydrocarbons recovered from a snow crab fished on the Scotian Shelf.*

more by laboratory-staff health concerns, an efficient solvent system of low toxicity and using hexane/isopropanol was introduced as early as 1978 (*43*). Originally intended for soft tissue, specifically brain, these systems have also been recently reevaluated for lipids of other origins including shellfish (*44, 45, 46*).

Finally, chemical methods for the assay of large and variably chlorinated materials are complex and very expensive in terms of modern equipment and technology (*47*). The overall process has not been simplified since 1992, except that greater use of stable isotopes and labelled reference material may be useful adjuncts (*48*) and chromatography is still the keystone of the analytical picture (*49*).

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### Problems with Sampling

At this point it is necessary to observe that picking up mussels at the seashore, off a buoy, or an oil rig is apt to yield irreproducible results due to age and size effects (*50*). Recent research suggests that in the long-term water depth is more critical than might be thought (*51, 52*). The difference between chronic exposure and spill exposure in terms of depuration rate is very large (*53, 54*). Surface water films may be different in hydrocarbon composition from ocean water (*23, 55*) and may affect intertidal samples. The migration of sentinel organisms is fortunately not a problem with mussels compared to lobsters (*48*) and snow crabs.

Only a light grade of crude petroleum is produced on the Scotian Shelf. Water-soluble fractions of most crude oils appear to be qualitatively similar, simplifying comparisons with literature material from highly contaminated areas such as the Baltic or North Sea. Our technologies however provide handling procedures for exposure trials with aquatic organisms (*20, 21, 56*) as well as analytical methods (*13, 14, 18, 57*).

The safety of consumers of marine and freshwater fishery products should always be of concern (*58*). The source of many fish and shellfish is beyond our control but off-flavor problems in aquaculture, which may be natural (*59*) or from industrial pollution, raises particularly sensitive issues of responsibility (*1*, 60).

## The Misunderstood Word "Tainting"

Unfortunately, the problem of "tainting" as applied to retail products for public consumption has become confused with environmental contamination. Tainting can be defined as "that concentration in water that will, after exposure of the test organism to the water, cause a change in flavor that can be detected by 50 percent of a population of assessors." This definition has been aptly demonstrated with the natural product 2,4-dichlorophenol (*61*). This chemical and some similar algal compounds are a problem for industries involved in cultivating and fishing prawns (shrimp) in Australia (*62*). Table I demonstrates that very small amounts of an unfamiliar chemical are required to produce an off-flavor effect. In contrast, the alkane hydrocarbons that are most obvious as a natural background in seawater are basically produced by algae on a worldwide basis (*26*). Unfortunately, these hydrocarbons are common in petroleum products such as fuels and lubricants in day to day use but fortunately have bland flavors and can safely go unnoticed in fishery products. It is important to distinguish such harmless water-soluble hydrocarbons from aromatic and polycyclic compounds possessing carcinogenic properties and giving quite another meaning to "tainting".

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## Chapter 7

# Effect of an Artificial Diet on Lipid, Free Amino Acid, and Carotenoid Composition of Green Sea Urchin Gonads

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Flavor is an important sensory attribute of sea urchin gonads. The flavor of gonads is also affected by dietary factors. Lipid fatty acids and carotenoids play an important role in the development of off-flavors during post-mortem period. In addition, free amino acids serve as a major taste-active component in urchin gonads. Hence, the effect of feeding of urchins initially on a *Laminaria* kelp followed by a grain-based diet on the total lipids and their fatty acids, free amino acids and carotenoids was evaluated. Eicospentaenoic acid was dominant in the lipids of urchins fed on an algal diet, but linoleic acid became dominant upon feeding on an artificial diet. Glycine was the major free amino acid throughout the feeding period and its contribution decreased with the progression of feeding. Carotenoid content, on the other hand, decreased from 23.2 to 9.2 mg/100 g tissue after feeding on the artificial diet. Thus, the role of flavor components and/or flavor precursors of urchin gonads is confirmed.

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Sea urchins belong to the marine invertebrate phylum Echinodermata (*1*), and their gonads have been favored by consumers because of their distinctive aroma and exceptional taste. Goands, the edible portion of sea urchin, are comprised of five lobes that are half-moon shaped and yellow-orange in color (*2*). Increasing demand for sea urchin gonads and a steady rise in their prices have led to worldwide intensification of sea urchin fisheries. However, wild sea urchin stocks constitute a limited resource and many are in decline as a result of over-harvesting. Hence, sea urchin aquaculture and gonad enhancement may create new opportunities in this area (*3*).

In general, kelp is the natural food for sea urchins and an abundant supply of it provides good gonad yield (*4*). However, the use of fresh algae is not always possible or profitable on a large scale. Therefore, an artificial diet designed especially for sea urchins is necessary for intensified echiniculture (*5*). The culture of sea urchins depends on the availability of a proper commercially produced feed that is as successful as the sea urchin's natural food. Therefore, in order to develop an economically viable sea urchin industry, it is important to evaluate the effect of feed on the quality of sea urchin gonads.

The assessment of gonad quality is a subjective pocess and is sometimes rather difficult to quantify. However, the general components of quality judgement mainly include consideration of gonad color, size, taste, texture and freshness. The quality of gonads, similar to other seafoods, undergoes rapid changes especially with respect to aroma and flavor characteristics of fresh products (*6*). It has been shown that the quality deterioration of unfrozen seafoods proceeds via biochemical processes in proteins and non-protein nitrogenous compounds during post-mortem period while sensory changes are primarily brought about by lipid oxidation in frozen seafoods.

The objective of the present study was to investigate the effect of feeding of sea urchins initially on a *Laminaria* kelp diet followed by a grain-based feed on the total lipids and their fatty acid composition. The effect of feed on free amino acid composition and carotenoid pigmentation of gonads of green sea urchin, *Strongylocentrotus droebachiensis*, was also investigated.

## Materials and Methods

Sea urchins were collected at a depth of 2 m at Bonavista Bay, Newfoundland, and were cultured in the Sea Urchin Research Facility (SURF) in the same area. Urchins were cultured in raceways using sea water to maintain similar salinity. The temperature was adjusted to simulate temperature effects in nature. Initially, urchins were fed on a *Laminaria* kelp diet for 3 weeks. After 3 weeks of algal feeding, urchins were harvested for analysis and this harvest was considered to be the control which indicates the week 0 harvest. Thereafter, a similar salinity. The temperature was adjusted to simulate temperature effects in nature. Initially, urchins were fed on a *Laminaria* kelp diet for 3 weeks. After 3 weeks of algal feeding, urchins were harvested for analysis and this harvest was considered to be the control which indicates the week 0 harvest. Thereafter, a grain-based feed formulation (Table I) was introduced and feeding was continued for 9 weeks. Urchins were harvested at 3, 6 and 9 weeks during experimental feeding of the artificial diet.

**Table I. Composition of the grain-based sea urchin feed**

| *Constituent* | *Content (Weight %)* |
|---|---|
| Soy meal | 19.6 |
| Wheat middling | 19.6 |
| Barley | 19.6 |
| Corn meal | 19.6 |
| *Laminaria longicruris* | 10.0 |
| Gelatin | 5.0 |
| Lecithin | 5.0 |
| Sodium alginate | 1.5 |

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Total lipids were extracted into chloroform/methanol by Bligh and Dyer (*7*) and then quantified. The fatty acid composition of lipids was determined by gas chromatography (GC) following their transmethylation, as described by Wanasundara and Shahidi (*8*). Fatty acid methyl esters (FAMEs) were prepared using 6% (v/v) sulfuric acid in methanol containing 15 mg of *t*-butylhydroquinone (TBHQ)} at 65 C for 15 h. FAMEs were extracted into hexane, dried and then dissolved in carbon disulfide and subjected to GC analysis. FAMEs were identified by comparing their retention times with those of authentic standard mixtures (Nu Check-Prep, Elysian, MN) and literature values (*9*, *10*).

Free amino acids were extracted into 6% perchloric acid and analysed using a Beckman 121 amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA). Total carotenoids content was determined by the method of McBeth (*11*). Carotenoids were extracted into acetone and then transferred into *n*-hexane. The absorption spectra were recorded at 400 to 600 nm using a spectrophotometer.

## Results and Discussion

### *Lipid and Lipid Fatty Acid Composition*

In general, lipids serve as a source of condensed energy, essential fatty acids and fat-soluble vitamins. In addition, they provide a reaction mechanism for the interaction of different food components and the generation of aroma compounds (*12*). Lipids may influence the flavor attributes of foods through aroma and aroma effects, flavor character, and flavor masking, as well as flavor release and development. Lipids are also known to play a critical role during processing of food thus affecting the stability of storage during storage since lipids are highly susceptible to oxidation. Although the total amount of lipid deposited is important, the fatty acid composition of lipids is critical because it may influence flavor and storage characteristics of products (*13*). One of the mechanisms through which lipids participate in aroma generation includes oxidation of long-chain polyunsaturated fatty acids. Thus, the level of fat deposition may influence both nutritional and organoleptic properties of a product (*14*).

The total lipid content of the sea urchin gonads is shown in Table II. The composition of the artificial feed (Table III) had a direct influence on the total lipid content. Thus, in the present study, the feed contained only 3.7% lipids and this factor brought about a significant decrease in the total lipids of gonads compared to week 0 when urchins were given a laminarian diet. Cook et al. (*15*) reported that the gonadal growth was very high when urchins were fed on protein- and lipid-rich artificial feeds compared to a macroalgal diet that resembled the urchin's preferred natural diet. In fact, higher amounts of lipid in dietary formulations may minimize protein utilization as a source of energy, thus improving the growth performance (*15*). However, the use of high energy diets containing up to 33% lipid has led to products having an oily texture and strong flavors, in addition to poor pigmentation. Furthermore, such a product may be susceptible to rancidity (*16*).

Fatty acid composition of sea urchin gonadal lipids is shown in Table IV. The major saturated fatty acids (SFA) of sea urchin lipids were 14:0, 16:0 and 20:0. The fatty acid 20:1n-15 was the dominant monounsaturated fatty acid (MUFA) on week 0 while 18:1n-9 became more dominant after feeding of sea urchins on the artificial feed. The major polyunsaturated fatty acids (PUFA) during the entire experimental feeding were 20:4n-6 (arachidonic acid, AA) and 20:5n-3 (eicosapentaenoic acid, EPA). However, the fatty acid 18:2n-6 (linoleic acid, LA) that contributed only 1.1% to the total fatty acid content on week 0 became dominant at the end of the experimental feeding. Thus, LA and EPA showed opposite patterns of variation with the progression of feeding. Moreover, there was a predominance of total content of PUFA in the fatty acid

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**Table II. Total lipid content of sea urchin gonads at 0, 3, 6 and 9 weeks after feeding the artificial diet**

| *Harvest time (weeks)* | *Lipid conten t(wet weight basis) (%)* |
|---|---|
| 0 | 4.7 (0.1) |
| 3 | 4.0 (0.2) |
| 6 | 4.5 (0.1) |
| 9 | 3.8 (0.2) |

Results are mean values of three samples. Values in parentheses are the standard deviation from means.

**Table III. Proximate composition of the artificial feed**

| Component | Weight (%) |
|---|---|
| Moisture | 14.3 (0.03) |
| Ash | 3.3 (0.1) |
| Total lipid | 3.7 (0.3) |
| Crude protein | 13.6 (0.3) |
| Carbohydrates | 66.7 (0.4) |

Results are mean values of three samples. Values in parentheses are the standard deviation from means.

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profile of sea urchin gonadal lipids throughout the feeding irrespective of the type of diet.

In general, fatty acid composition of tissues reflects that of their dietary sources. For example, Floreto et al. (*17*) showed that fatty acid profiles of sea urchins fed on a seaweed diet resembled that seaweeds. However, certain fatty acids such as 16:4n-3, 20:4n-6, 20:5n-3 and 20:1n-11 have been reported to be dominant in sea urchin tissues even if these fatty acids are undetectable or present only in very small amounts in their diets. This evidence suggests that sea urchins are capable of synthesizing certain fatty acids from their lower fatty acid precursors.

The fatty acids of gonadal lipids in week 0 were typically similar to those of marine species with a dominance of 16:0 and 20:5n-3 (*18*). Although 22-6n-3 (docosahexaenoic acid, DHA) is also a typical fatty acid in marine lipids, it contributed a maximum of 1.4% on week 0 to the total fatty acids after which its content decreased with the progression of feeding on the artificial diet.

The major SFA in sea urchin gonadal lipids were 14:0 and 16:0, in accordance with the published data on fatty acid composition of sea urchin (*9*, *19*). Thus, the artificial diet did not have a significant effect on both of these major SFA. With respect to MUFA, 20:1n-15 was the dominant MUFA in sea urchin lipids on week 0. Takagi et al. (*9*) stated that sea urchins may contain 20:1n-15 up to 11% of their fatty acid content. However, marine animals may contain 20:1n-15, but at much lower levels not exceeding 0.2% of the total fatty acid content (*20*). Furthermore, it has been reported that 20:1n-15 was not found in seaweeds, the natural diet of sea urchins (*21*). This fatty acid was also absent in the artificial diet employed in this study.

**Table IV. Fatty acid composition (weight %) of total lipids from sea urchin gonadal lipids at 0 and 9 weeks of feeding the artificial diet**

| *Fatty acid* | *Week 0* | *Week 9* |
|---|---|---|
| 14 : 0 | 9.4 (0.1)$^{a}$ | 6.7 (0.1)$^{b}$ |
| 16 : 0 | 11.1 (0.1)$^{a}$ | 10.5 (0.1)$^{b}$ |
| 18 : 0 | 2.2 (0.04)$^{a}$ | 1.2 (0.2)$^{b}$ |
| 20 : 0 | 2.9 (0.1)$^{a}$ | 4.3 (0.4)$^{b}$ |
| 16 : 1 n-9 | 4.8 (0.1)$^{ac}$ | 4.8 (0.1)$^{c}$ |
| 16 : 1 n-7 | 1.5 (0.1)$^{a}$ | 1.8 (0.1)$^{b}$ |
| 18 : 1 n-9 | 1.8 (0.1)$^{a}$ | 4.9 (0.1)$^{d}$ |
| 18 : 1 n-7 | 3.6 (0.1)$^{a}$ | 3.6 (0.1)$^{a}$ |
| 20 : 1 n-15 | 7.5 (0.2)$^{a}$ | 3.2 (0.1)$^{b}$ |
| 20 : 1 n-9 | 4.0 (0.03)$^{a}$ | 1.4 (0. 03)$^{c}$ |
| 20 : 1 n-7 | 2.2 (0.1)$^{a}$ | 4.6 (0.05)$^{c}$ |
| 22 : 1 n-11 | 2.9 (0.04)$^{a}$ | 0.2 (0.03)$^{c}$ |
| 22 : 1 n-9 | 0.4 (0.03)$^{a}$ | 1.6 (0.1)$^{b}$ |
| 16 : 2 n-6 | 0.5 (0.03)$^{a}$ | 1.2 (0.04)$^{c}$ |
| 16 : 4 n-6 | 1.9 (0.1)$^{a}$ | 1.1 (0.03)$^{c}$ |
| 18 : 2 n-6 | 1.1 (0.1)$^{a}$ | 15.7 (0.1)$^{d}$ |
| 18 : 3 n-6 | 1.4 (0.03)$^{a}$ | 0.4 (0.04)$^{b}$ |
| 18 : 3 n-3 | 1.3 (0.04)$^{a}$ | 1.8 (0.2)$^{b}$ |
| 18 : 4 n-3 | 3.8 (0.03)$^{a}$ | 1.7 ( 0.1)$^{c}$ |
| 20 : 2 Δ5,11 | 1.8 (0.1)$^{a}$ | 2.1 (0.04)$^{ac}$ |
| 20 : 2 n - 6 | 1.7 (0.1)$^{a}$ | 5.2 (0.1)$^{c}$ |
| 20 : 3 n-6 | 0.4 (0.1)$^{a}$ | 1.1 (0.1)$^{c}$ |
| 20 : 4 n-6 | 7.0 (0.1)$^{a}$ | 5.0 (0.1)$^{c}$ |
| 20 : 3 n-3 | 1.7 (0.2)$^{a}$ | ND |
| 20 : 4 n-3 | 1.2 (0.02)$^{a}$ | 1.8 (0.03)$^{c}$ |
| 20 : 5 n-3 | 16.3 (0.1)$^{a}$ | 8.4 (0.1)$^{d}$ |
| 22 : 5 n-6 | 0.2 (0.1)$^{a}$ | 0.3 (0.04)$^{a}$ |
| 22 : 5 n-3 | 0.6 (0.1)$^{a}$ | 0.2 (0.03)$^{b}$ |
| 22 : 6 n-3 | 1.4 (0.1)$^{a}$ | 0.1 (0.1)$^{b}$ |

Results are mean values of three samples. Values in parentheses are the standard deviations from the means. Values in each row with the same superscript are not significantly different ($p>0.05$) from one another. ND = not determined.

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On week 0, 20:5n-3 content was quite high in sea urchin lipids. It has been shown that in sea urchins fed on seaweeds containing 18:2n-6 and 20:4n-6, these fatty acids may partly substitute the membranes for their linolenic-family fatty acid, mainly 20:5n-3 (*17*). This discovery is further supported by the results obtained for sea urchin gonadal lipids in the present study. The artificial feed contained approximately half of its total fatty acids in the form of 18:2n-6 and very little of 20:5n-3 (Table V). After introducing the artificial diet, the content of 18:2n-6 increased while that of 20:5n-3 decreased. Hence, the diet of sea urchins may exert a major effect on their fatty acid composition.

**Table V. Fatty acid composition (weight %) of grain-based sea urchin feed**

| *Fatty acid* | *Weight %* |
|---|---|
| 16 : 0 | 16.7 (0.2) |
| 18 : 0 | 7.6 (0.4) |
| 18 : 1 n-9 | 18.8 (0.2) |
| 18 : 2 n-6 | 49.1 (0.2) |
| 18 : 3 n-3 | 5.2 (0.2) |
| 20 : 5 n-3 | 0.1 (0.1) |
| 22 : 6 n-3 | 0.2 (0.1) |

Results are mean values of three samples. Values in parentheses are the standard deviations from the means.

### *Free amino acid composition*

The taste of sea urchin gonads, in general, is dependent on extractive water-soluble and low-molecular weight compounds. These extractive components may be either nitrogenous or non-nitrogenous in nature (*22*). Free amino acids (FAA) constitute one of the major nitrogenous extractive components (*23*). Marine invertebrates characteristically contain a high intracellular concentration of FAA although the composition of FAA profile may vary among different species (*24*). Almost all FAA have some sweetness, bitterness, sournes or umami taste (taste of monosodium glutamate). In fact, FAA are responsible for the distinctive taste of many marine and other foods (*25*). Glycine, alanine and leucine were the major amino acids in sea urchin gonads (*26*).

The free amino acid profile of sea urchin gonads is shown in Table VI. Glycine was the dominant amino acid and contributed 15.3-57.1% to the total FAA content. The relatie proportion of glycine was more than 50% of the total free amino acid content on week 0 when urchins were fed on a laminarian diet. Komata et al. (*26*) reported that glycine was the dominant FAA in the sea urchins *Strongylocentrotus pulcherrimus,* ranging from 35-41% of total FAA. Lee and Haard (*27*) reported that glycine represented 18-60% of the total FAA

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**Table VI. Free amino acid content (mg/g dry weight) of sea urchin gonads**

| *Amino acid* | *Week 0* | *Week 9* |
|---|---|---|
| Alanine | 2872 (77)$^a$ | 4239 (121)$^c$ |
| α-aminoadipic acid | 68 (5)$^a$ | 277 (43)$^b$ |
| Arginine | 180 (3)$^a$ | 13701 (211)$^d$ |
| Asparagine | 13 (1)$^a$ | 1843 (78)$^d$ |
| Aspartic acid | 73 (8)$^a$ | 471 (9)$^c$ |
| Cystathionine | 115 (2)$^a$ | 1128 (29)$^b$ |
| Glutamic acid | 874 (12)$^a$ | 2321 (987)$^d$ |
| Glutamine | 647 (34)$^a$ | 4389 (123)$^c$ |
| Glycine | 11751 (223)$^a$ | 28301 (734)$^b$ |
| Histidine | 100 (6)$^a$ | 5128 (132)$^d$ |
| Hydroxyproline | 124 (11)$^a$ | 1800 (99)$^c$ |
| Isoleucine | 313 (11)$^a$ | 9866 (113)$^d$ |
| Leucine | 370 (23)$^a$ | 16854 (267)$^d$ |
| Lysine | 356 (7)$^a$ | 11681 (198)$^b$ |
| Methionine | 68 (4)$^a$ | 1604 (97)$^d$ |
| Phenylalanine | 164 (2)$^a$ | 7114 (103)$^d$ |
| Proline | 140 (10)$^a$ | 617 (10)$^d$ |
| Sarcosine | 332 (14)$^a$ | 71 (12)$^b$ |
| Serine | 316 (5)$^a$ | 4209 (68)$^d$ |
| Taurine | 214 (2)$^a$ | 792 (27)$^c$ |
| Threonine | 521 (27)$^a$ | 6676 (103)$^c$ |
| Tryptophan | 264 (11)$^a$ | 2044 (58)$^c$ |
| Tyrosine | 217 (16)$^a$ | 16493 (497)$^c$ |
| Valine | 273 (16)$^a$ | 12668 (3270)$^d$ |

Results are mean values of three samples. Values in parentheses are the standard deviations from the means. Values in each row with the same superscript are not significantly different ($p>0.05$) from one another.

in *S. droebachiensis*. However, Cruz-Garcia et al. (*28*) found that in the sea urchin *Paracentrotus lividus,* glycine was not the dominant FAA although it contributed a considerable amount to itstotal content of amino acids.

Glycine and alanine are known to contribute to the sweetness of gonads while valine and glutamine are responsible for bitterness and umami taste, respectively (*29*). Glycine and glutamine are both "taste-active" in sea urchins, irrespective of their quantity (*29*). In addition, alanine, argnine, glutamic acid, lysine and methionine are important to the taste even though some may be present in small quantities (*27*). Valine and methionine are taste-active only in sea urchin gonads (*29*). Thus, FAA are one of the major extractive components responsible for the specific taste sea urchin gonads.

Sea urchins' FAA pools were greatly affected by feeding on the artificial diet (Table VII). The FAA content was obviously lower in week 0 when urchins were fed *Laminaria* as compared to other times when urchins were fed on the artificial diet during which the total FAA content increased by many times in week 3 followed by a decrease in weeks 6 and 9. Thus, total FAA content in the gonads increased from 20.6 mg/g dry weight to 180.6 mg/g dry mass in week 3. Although glycine remained the dominant amino acid even after introducing the artificial diet, its proportion was greatly reduced while the proportion of a few other amino acids such as arginine, lysine, leucine, tyrosine, valine, isoleucine and phenylalanine increased considerably.

## *Carotenoid pigmentation*

The total carotenoid content (on a dry weight basis) of sea urchin gonads is shown in Table VIII. In the sea urchin *S. droebachiensis,* carotenoids were mainly concentrated in the gonadal tissues and their total content was reduced significantly with the progression of feeding period. The total carotenoid content decreased from 23.2 on week 0 to 9.2 mg/100 g tissue on week 9; thus reflecting a decrease of more than 50% by the end of 9 weeks of feeding. The total carotenoid content of the artificial diet used in our study was 0.38 mg/100 g feed on a dry weight basis. In general, animals cannot synthesize carotenoids *de novo* and are therefore dependent upon dietary carotenoids (*30*). Since the feed provided only a very low amount of carotenoids a significant reduction in the carotenoid content of the sea urchin tissues occurred. Thus, supplementation of the artificial diet with carotenoids appears necessary. These carotenoids not only provide a desirable color to the product, but also protect their lipid constituents against oxidation allowing the characteristic flavor of urchins to be recealed.

**Table VII. Content of total free amino acids in sea urchin gonads on a dry weight basis**

| *Harvest time(weeks)* | *Content (dry weight basis) (mg/g tissue)* |
|---|---|
| 0 | 20.6 |
| 3 | 180.6 |
| 6 | 153.8 |
| 9 | 154.6 |

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**Table VIII. Total carotenoid content (mg per100g) in sea urchin gonads**

| *Harvest time (weeks)* | *Carotenoid content* |
|---|---|
| 0 | 23.2 (0.04)$^a$ |
| 3 | 20.1 (0.2)$^b$ |
| 6 | 11.0 (0.2)$^c$ |
| 9 | 9.2 (0.1)$^d$ |

Results are mean values of three samples. Values in parentheses are the standard deviations from the means. Values in each column with the same superscript are not significantly different ($p>0.05$) from one another

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## Conclusions

A significant effect on the total lipids and their fatty acid composition, free amino acid composition and carotenoid content of sea urchin gonads was observed upon feeding on an artificial diet. All parameters tested were important in flavor generation of sea urchin gonads. Thus, the type and quality of feed plays an important role in the flavor of sea urchin gonads since flavor components and/or flavor precursors are affected by the feed. The artificial feed employed in this study was deficient in nutritionally important PUFA that are a major component in their natural diet. Hence, EPA content was gradually decreased with the progression of feeding. The artificial diet was also deficient in carotenoid pigments thus causing a significant reduction in carotenoid content of gonads. In general, carotenoids are effective antioxidants and act as protective agents against lipid oxidation. Therefore, inclusion of appropriate carotinoids in the feed may be necessary. In addition, carotenoids will impart a desirable color to sea urchin gonads. Hence, development of a suitable feed for sea urchin aquaculture requires careful consideration of the selection of the ingredients in order to delay the onset of off-flavor formation in the gonads.

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## Chapter 8

# Storage of Off-Flavors in Adipocytes of Salmon Muscle

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Understanding the storage locations of off-flavors in tissues of aquatic organisms is an important step in finding solutions of effectively removing and depurating off-flavors from fish and shellfish in the aquaculture industry. Experiments were carried out in exposing Atlantic salmon to the water-soluble fraction (WSF) of crude petroleum hydrocarbons, and in depurating the hydrocarbon off-flavors in exposed fish in clean water. Muscle tissue, adipocytes isolated from muscle tissue, and subdermal fat were collected and analyzed for WSF hydrocarbon content at the end of exposure and during depuration. Both white muscle tissue and adipocytes were heavily tainted with WSF hydrocarbons at the end of exposure, but adipocytes in the muscle tissue accounted for about 55% of accumulated WSF hydrocarbons. The non-adipocyte portion of muscle tissue was freed of WSF hydrocarbons rapidly during depuration in clean water, while the depuration of WSF hydrocarbons from adipocytes was much slower. As a result, adipocytes became the predominant storage sites for the remaining WSF hydrocarbons in muscle tissue after 4 days of depuration in clean water. The accumulation and depuration of WSF hydrocarbons from subdermal fat were similar to those from adipocytes.

# INTRODUCTION

Numerous investigations have been conducted to study the uptake and depuration of off-flavors, including petroleum hydrocarbons, in aquatic organisms (1, 2, 3). It is generally accepted that lipids play an important role in the accumulation and retention of off-flavors (4). Lean fish and shellfish, such as cod and scallop, accumulated small amount of the water-soluble fraction (WSF) of petroleum hydrocarbons during exposure and were freed of the tainting WSF hydrocarbons rapidly when returned to clean water (5, 6). On the other hand, farmed salmon, a fatty fish species, accumulated larger quantity of hydrocarbon pollutants under similar conditions and took months to depurate all of the tainting WSF hydrocarbons, particularly polyaromatic hydrocarbons (4). These lipid related species-specific characteristics have also been observed for rainbow trout and cyprinids (7, 8). The role of lipids in the uptake and depuration of off-flavors was also important in freshwater species (9). The fatter channel catfish (>2.5% muscle fat) accumulated nearly three times more 2-methylisoborneol than lean fish (<2% muscle fat). In clean water, the accumulated off-flavor in leaner fish was depurated much faster (8 h) than that in fatter fish (48 h). In fact, several approaches have been taken in considering the effect of tissue lipid content on the accumulation of organic pollutants. These include octanol-water partitioning coefficients (10, 11) and normalization to a lipid basis (12).

It was a common practice that studies on the accumulation and retention of off-flavors or organic contaminants in aquatic organisms were usually carried out by analyzing one of the following samples from target orgamisms: entire organism, whole tissue, part of tissue, or individual internal organ. Therefore, the results on off-flavor obtained from general practice should only be applied to the specific tissue analyzed. The actual accumulation and retention of off-flavors in specific cell compartments of tissues, particularly adipocytes, have rarely been studied and remain largely unknown.

The muscle tissue of aquatic organisms is mainly composed of muscle cells and to a much smaller extent adipocytes as well as other tissue components. Atlantic salmon (*Salmo salar*) is a fatty fish species and contains up to 16% total lipids in muscle tissue. Because of its high lipid content, it is an ideal candidate for the investigation of accumulation and retention of WSF hydrocarbons in adipocytes. The objective of this work is to reveal the role of adipocytes in the muscle tissue of Atlantic salmon in relation to the uptake, release and retention of WSF hydrocarbons by the whole of the muscle tissue.

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# MATERIALS AND METHODS

## Exposure of Atlantic Salmon to WSF Hydrocarbons and Depuration in Clean Seawater

Flotta North Sea crude oil was stirred with seawater in a ratio 1:99 (v/v) for 24 h followed by 48 h settlement to obtain a WSF hydrocarbon stock solution. This stock solution was diluted with fresh seawater to obtain an exposure level of 0.2 ppm of total WSF hydrocarbons in water, which was maintained for 96 h in the exposure tank. Eighteen market-sized Atlantic salmon, *Salmo salar,* were used for the exposure studies and six control fish were kept in clean seawater under the same conditions. At the end of exposure, three fish were killed for the sampling of muscle tissue, from which adipocytes were immediately isolated. The remaining fish were depurated in clean seawater and fish (three each time) were taken on days 1, 4, 10, and 20 for analyses of WSF hydrocarbons in white muscle, adipocytes and subdermal fat. Three of the six control fish were sampled on days 0 and 10, respectively.

## Isolation of Apipocytes and Analyses of WSF Hydrocarbons

Fish were killed with a blow on the head and the gut cavities were cut open along the middle of the abdomen. A cylinder shaped portion of dorsal white muscle (2x2x10 cm) was excised adjacent to the vertebrae, 2 cm from the head (Figure 1). The dorsal white muscle portions thus dissected from each of the three fish were cut into several pieces, pooled, and separated into two portions. One portion was used for WSF hydrocarbon analysis and the other portion for the determination of total lipids and lipid storage in the myosepta of the dorsal white muscle tissue (13). Strips of belly flaps, about 2 cm in width, were cut along one side of the half fillet from the pectoral fin to the pelvic fin (Figure 1). The skins of the cut belly flaps and bundles of muscle fibres were then removed, leaving belly flaps enriched in connective tissue from the three fish which were pooled. Subdermal fat was obtained by scraping off the fat layer from the muscle surface after removing the fish skin. All dissection work was performed in a cold room at 5 °C.

About 9 g of the dissected belly flaps were immediately placed in a Petri dish containing Krebs-Ringer phosphate buffer with 1% albumin (bovine, fraction V, Sigma Chemical Co., St. Louis, MO). The tissues were cut into small pieces

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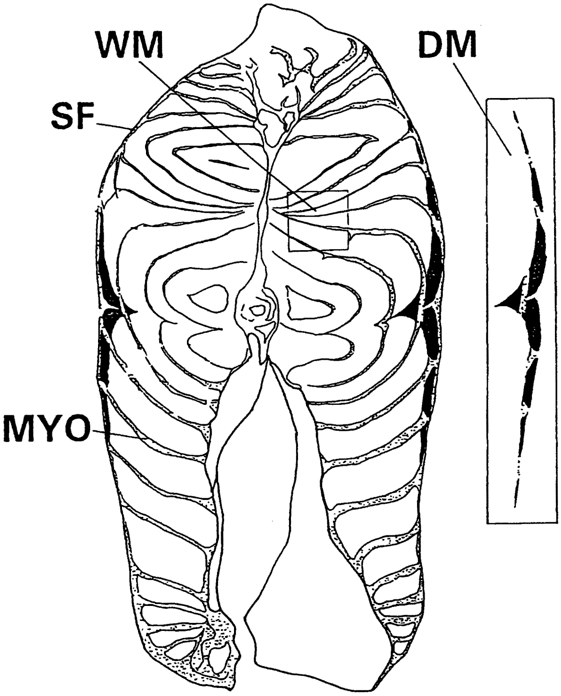

*Figure 1. Cross section of salmon body. WM – dorsal white muscle; DM – dark muscle, MYO – myosepta; SF – subdermal fat tissue.*

in the Petri dish and added to a 4 oz Nalgene plastic bottle containing 22 mL of 1% albumin in phosphate buffer plus 180 mg collagenase (type II, Sigma). The digestion processes applied to tissue from the belly flaps and the technique for discrimination between isolated adipocytes and free fat have been described elsewhere (14). The resulting dialysis tubing containing sections of the isolated adipocytes, the free fat layer and the buffer solution was immediately frozen at –35 °C. The adipocyte block of ice was then cut off and stored at –35 °C until analysis.

The WSF hydrocarbons in the dorsal white muscle and the isolated adipocyte block of ice were recovered by steam distillation technique and quantified by GLC-FID (15).

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### Lipid Analyses

The lipid content of dorsal white muscle was determined by following the method of Bligh and Dyer (16). The quantification of lipid storage in myosepta of the white muscle tissue was determined by following the procedures described by Zhou et al. (13).

## Results and Discussion

### Adipocyte Distribution in Muscle Tissue

Loose connective tissue is widely distributed in the fish body and is characterized by a relative dominance of resident cells. In Atlantic salmon muscle, white streaks of connective tissue, mainly myosepta, could be easily seen against the pink-yellowish muscle background (Figure 1). Our previous histological studies have shown that in muscle tissue proper adipocytes were mainly distributed in myosepta and the connective tissue and associated adipocytes decreased sharply from the belly flap region to the leaner dorsal white muscle (Figure 1). Lipid droplets occurred in the connective tissue surrounding dark muscle fibers, but were only occasionally around dorsal white muscle fibers. Finely dispersed intracellular lipid droplets were present in dark muscle cells but were not observed in dorsal white muscle cells (14).

## Lipids in Connective Tissue Myosepta

Lipids extracted from muscle tissue using the chloroform-methanol solvent system (16) mainly contain triglycerides, free fatty acids, sterols and phospholipids. Both phospholipids and sterols are major components of tissue cell membrane. The average total lipid content and membrane lipid content of dorsal white muscle in salmon used in this study were 4.0% and 0.6% (w/w), respectively. Quantitative determination of lipid classes in muscle tissue with and without myosepta (13) showed that 38.9% of average total lipids found in dorsal white muscle of all exposed fish was located in myosepta of that dorsal white muscle. This strongly supports our previous histological findings that adipocytes in dorsal white muscle are mainly distributed in myosepta of white muscle of Atlantic salmon (14).

## Uptake and Depuration of WSF Hydrocarbons in Adipocytes and Muscle Tissue

WSF hydrocarbons of Flotta North Sea crude oil were a mixture of various alkylated aromatic hydrocarbons, primarily alkylated benzenes. Table 1 shows the total WSF hydrocarbons accumulated in adipocytes and in whole dorsal white muscle at the end of 96 h exposure and during the 20 day depuration period. After exposing to 0.2 ppm WSF hydrocarbons in water for 96 h, adipocytes accumulated 59.5 ppm of total WSF hydrocarbons, while in dorsal white muscle, only 4.2 ppm of WSF hydrocarbons was detected. Dorsal white

**Table 1. Release of WSF Hydrocarbons from Whole Dorsal White Muscle and from Adipocytes Isolated from Muscle During 20 Day Period of Depuration.**

| | *Adpocytes* | | *Whole Dorsal White Muscle* | |
|---|---|---|---|---|
| *Depuration Time (day)* | *Tainting WSF-HC (ppm)* | *Percent Remaining (%)* | *Tainting WSF-HC (ppm)* | *Percent Remaining (%)* |
| 0 | 59.5 | 100 | 4.20 | 100 |
| 1 | 48.1 | 81 | 1.87 | 45 |
| 4 | 27.3 | 46 | 0.96 | 23 |
| 10 | 18.5 | 31 | 0.71 | 17 |
| 20 | 10.8 | 18 | 0.22 | 5 |

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muscle in Atlantic salmon taken at the end of exposure contained 4.5% of total lipids and 0.6% of membrane polar lipids (phospholipids and sterols). Assuming that membrane lipids are composed of only phospholipids and sterols and all other neutral lipid components are associated with adipocytes or lipid sacs, 86% of the 4.5% of total dorsal white muscle lipids was in the form of adipocytes or lipid sacs. Because tainting WSF hydrocarbon content in adipocytes is 14 times higher than that in whole dorsal white muscle, the calculated amount of WSF hydrocarbons in adipocytes accounted for 54% of total tainting WSF hydrocarbons in the whole dorsal white muscle at the end of exposure period (Figure 2). After subtracting the WSF hydrocarbons stored in adipocytes from the total tainting WSF hydrocarbons in dorsal white muscle, the non-adipocyte portion of the dorsal white muscle, predominantly white muscle cells, was calculated to have taken up 2.0 ppm of WSF hydrocarbons at the end of exposure, an amount only ten times higher than the WSF hydrocarbon concentration in the exposure water.

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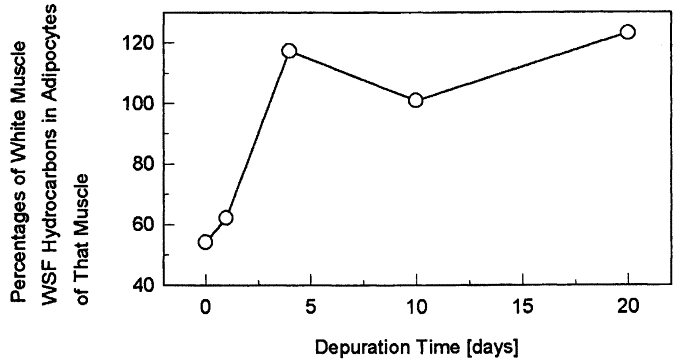

*Figure 2. Changes in calculated percentages of tainting WSF hydrocarbons in adipocytes of dorsal white muscle accounting for the total tainting WSF hydrocarbons in the same muscle tissue.*

Tainting WSF hydrocarbons were released from tainted fish into clean water during depuration. Compared with adipocytes, much faster depuration rate of WSF hydrocarbons from whole dorsal white muscle was observed during the 20 day depuration period (Table 1). After one day of depuration in clean water, 55% of tainting WSF hydrocarbons in whole dorsal white muscle was released, while only 19% of reduction in WSF hydrocarbons was observed in adipocytes (Table 1). The release of WSF hydrocarbons from the non-adipocyte portion of the whole white muscle would be much faster when the effect of adipocytes was excluded. Upon 20 day depuration in clean water, a very low concentration of WSF hydrocarbons (0.22 ppm) was detected in whole dorsal white muscle, while in adipocytes the tainting WSF hydrocarbons were still very high (10.8 ppm). In fact, this small quantity of remaining WSF hydrocarbons (0.22 ppm) in white muscle was actually stored in adipocytes (Figure 2). It would probably take months to depurate all of the tainting WSF hydrocarbons from adipocytes.

Salmon muscle tissue is composed of various cell components and intercellular fluid. The calculated percentage of WSF hydrocarbons in adipocytes accounting for the total WSF hydrocarbons in dorsal white muscle increased rapidly during the 20 day depuration period (Figure 2). After only 4 days of depuration, the non-adipocyte portion of the muscle tissue was freed of tainting WSF hydrocarbons and adipocytes became the predominant storage site of WSF hydrocarbons in dorsal white muscle (Figure 2).

Figure 3 compares the depuration of tainting WSF hydrocarbons from adipocytes and from subdermal fat. Both subdermal fat and adipocytes accumulated very high concentration of WSF hydrocarbons at the end of 96 h exposure and the tainting WSF hydrocarbons were released in a similar pattern. Subdermal fat contained 74.5 ppm of WSF hydrocarbons at the end of exposure and 8.0 ppm of WSF hydrocarbons after 20 day depuration. Subdermal fat tissue is composed of adipocytes, blood vessels, and connective tissue. The subdermal fat was obtained by scraping off the fat tissue layer after skin removal and contained little non-fat material. The higher level of accumulated WSF hydrocarbons in subdermal fat at the end of exposure could be attributed to its structural location in the fish body in which WSF hydrocarbons could be partitioned into adipocytes not only from blood vessel but also directly from the skin. Our previous histological studies have shown that the subdermal fat tissue of Atlantic salmon is predominantly composed of adipocytes. Therefore, it is expected that both the isolated adipocytes and the subdermal fat tissue would present the same characteristics in the uptake of WSF hydrocarbons from seawater and their subsequent depuration from those two tainted samples.

The depuration of individual WSF hydrocarbons was dependent on their molecular structure, i.e., their alkyl substitutions, number of aromatic rings, and to a larger extent their storage sites in the muscle tissue. All the WSF hydrocarbons in dorsal white muscle were released faster than those in the adipocytes. Figure 4 shows the depuration of benzene, toluene, and ethylbenzene & xylene from adipocytes and from dorsal white muscle during

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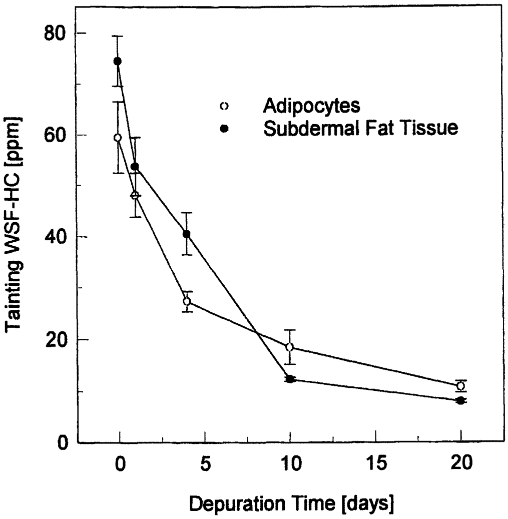

*Figure 3. Depuration of tainting WSF hydrocarbons of Flotta North Sea crude oil from the isolated adipocytes and the subdermal fat of market size Atlantic salmon. The fish were exposed to 0.2 ppm WSF hydrocarbons for 96 h.*

the 20 day depuration period. Benzene in dorsal white muscle was completely depurated within only one day (Figure 4.13a’), while it took 4 days to be depurated from adipocytes (Figure 4.13a). One methyl alkylation on the ring (toluene) required 4 days and 10 days respectively for the complete depuration from dorsal white muscle (Figure 4.13b’) and from adipocytes (Figure 4.13b). The depuration rate of ethylbenzene and xylene was significantly lower for both adipocytes and dorsal white muscle as alkylation on the ring increased (Figure 4.13c and c’).

The large difference found in the accumulation and release of WSF hydrocarbons between adipocytes and white muscle cells confirms that the species-dependent characteristics with respect to the accumulation and retention of hydrocarbons are actually dependent on both the tissue lipid content and the lipid storage format. Our present study on the separation of adipocytes from Atlantic salmon muscle tissue and the subsequent analysis of the tainting WSF hydrocarbons in this specific compartment of the tissue revealed that adipocytes

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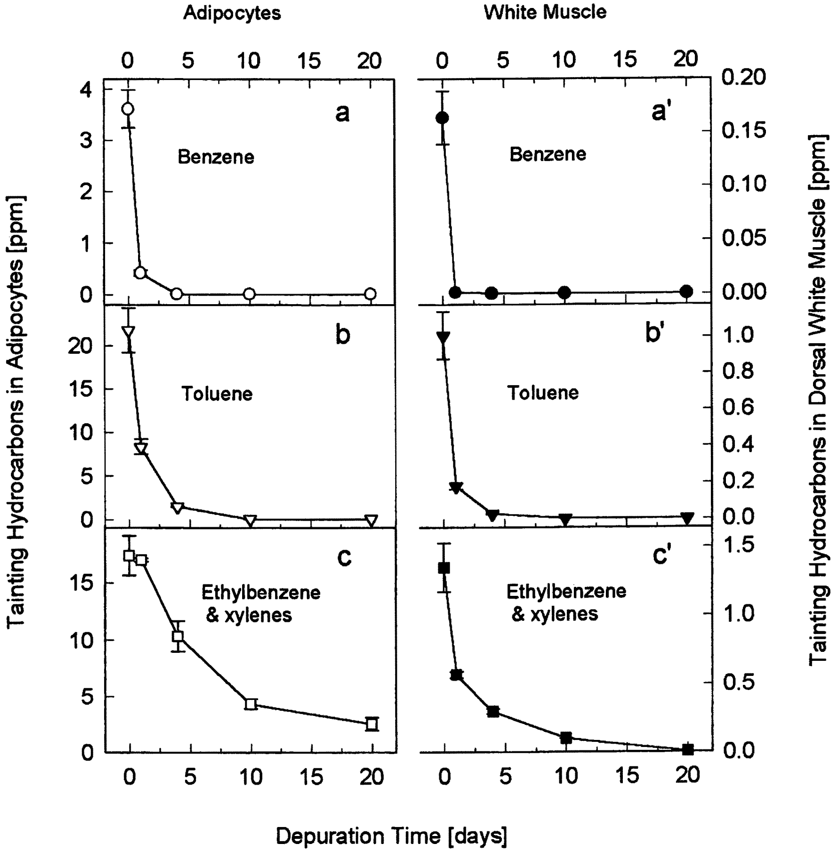

*Figure 4. Release of individual or groups of WSF hydrocarbons from adipocytes and dorsal white muscle during 20 day depuration period.*

in salmon muscle tissue do play an important role in controlling the uptake and release of hydrocarbons. The WSF hydrocarbons accumulated in the tissue were primarily stored in the adipocytes during long-term exposure and were also retained by the adipocytes during the depuration in clean seawater.

The accumulation and release of hydrocarbons in the tissue is most probably a passive process of partitioning of the hydrocarbons between the exposure water and the tissue lipids. The actual accumulation and release processes of WSF hydrocarbons into and from the muscle tissue are probably composed of several partitioning and diffusion steps before the WSF hydrocarbons finally reaches the lipid droplets in the adipocytes.

The non-adipocyte portion of white muscle is mainly composed of water, but the accumulated WSF hydrocarbons are most probably associated with the cell membrane polar lipids or the hydrophobic groups of proteins and glycolipids. The association of WSF hydrocarbons with these polar components is expected to be looser than their association with triacylglycerol lipid in the adipocytes where they are in true solution. Some of the WSF hydrocarbons in the non-adipocyte portions are presumably free to move around if they are merely associated with tissue fluids such as plasma. This compartment of WSF hydrocarbons can be quickly transported to the gills or to the liver. Thus they will be more readily released into the clean water column or metabolised by the liver (Thomas and Rice 1981). The loose association of WSF hydrocarbons with the components in the muscle cells and their presence in tissue fluids are probably the causes for the sharp release of tainting WSF hydrocarbons, mostly lower molecular weight aromatics, from white muscle in the early stage of depuration.

In conclusion, adipocytes in the white muscle tissue of Atlantic salmon were found to be the principal cell compartments for the storage and retention of WSF hydrocarbons, particularly after the initial rapid discharge of the accumulated WSF hydrocarbons from the muscle cells during the depuration periods. The role of adipocytes in the storage and retention of other xenobiotics may also be applicable to various other organic pollutants due to their similarity in hydrophobicity. This key role of adipocytes would also be expected to occur in various other aquatic organisms, particularly those with high lipid content.

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# Chapter 9

# Ecophysiology of Cyanobacteria: Implication for Off-Flavor Management in Pond Aquaculture

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The most prominent off-flavors in commercial catfish culture ponds are caused by cyanobacteria. Potential control methods must consider the environmental conditions and physiological attributes of cyanobacteria that favor their dominance of phytoplankton communities in hypertrophic aquaculture ponds. Selective pressures that lead to the dominance of cyanobacteria in hypertrophic aquaculture ponds include elevated water temperature, restricted light penetration caused by high algal turbidity, water column stability (stratification), high total nutrient concentrations, and low carbon dioxide concentration. Physiological attributes of cyanobacteria that contribute to dominance of phytoplankton communities include buoyancy regulation, accessory pigments, nitrogen fixation, high affinity for carbon dioxide, active transport of bicarbonate, secretion of allelopathic chemicals, and large size and mucopolysaccharide coatings rendering filaments and colonies resistant to grazing by zooplankton. Current configuration and management of ponds constrain opportunities for successful manipulation of phytoplankton community composition through conventional physical, chemical and biological approaches.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Fish cultured in semi-intensive ponds derive a majority of their nutrition from nutrient-dense manufactured feeds that are provided at rates sufficient to provide good growth of fish stocked at high density. Results from a wide variety of culture systems, species, and water types indicate that a minority (usually 25-30%) of nutrients applied in feeds are incorporated into harvested fish biomass. The remainder is released to the pond environment, stimulating luxuriant growth of plants, primarily phytoplankton. As a result, large standing crops of phytoplankton develop in semi-intensive aquaculture ponds, particularly during summer when conditions are favorable for plant growth.

As phytoplankton standing crop increases, light replaces nutrients as the primary factor regulating growth. Light-limitation also places a strong selection pressure on phytoplankton community structure, favoring species that are adapted to low irradiance. As a group, cyanobacteria are diverse and distributed through a wide range of habitats on the planet. They have developed a broad diversity of strategies to surmount severe constraints to growth and survival in extreme environments. Cyanobacteria are also extremely plastic in their response to environmental changes. Many cyanobacteria possess physiological attributes, primarily the ability to regulate position in the water column, that confers selective advantage over species that are less well adapted to low irradiance. Thus, cyanobacteria often dominate the phytoplankton communities of hypertrophic waters, forming the principal constituents of low-diversity communities.

Cyanobacteria are undesirable in aquaculture ponds for many reasons (*1*). Some cyanobacteria may produce substances that are toxic to zooplankton, cultured fish, and domestic animals or livestock. Cyanobacteria tend to form unstable monocultures that are subject to spectacular collapse. Cyanobacteria grow slowly and so photosynthetic oxygen production per unit biomass is lower than other phytoplankton. Some species form unsightly surface scums that restrict light penetration and thereby photosynthesis. Finally, and to the point of this symposium, some species produce odorous metabolites that impart an objectionable flavor to cultured fish.

The prominent off-flavors in cultured channel catfish are associated with methylisoborneol (MIB) produced by *Oscillatoria perornata* and geosmin produced by some species of *Anabaena*. As such, this review will emphasize the ecophysiology of *Oscillatoria* and *Anabaena*. Specifically, the review will concentrate on the physical, chemical, and biological characteristics of hypertrophic fish ponds that function to favor dominance of phytoplankton communities by *Oscillatoria* or *Anabaena*. The unique physiological attributes of cyanobacteria that confer selective advantage in the face of resource limitation, and thereby favor their dominance of the phytoplankton communities of hypertrophic aquaculture ponds, will be highlighted. Finally, opportunities for manipulation of the phytoplankton community composition will be discussed with respect to the physical, chemical,

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and biological factors favoring cyanobacterial dominance and with emphasis on techniques that reduce their selective advantage.

## Cyanobacteria in the Phytoplankton Communities of Semi-intensive Aquaculture Ponds

Although the practice is widespread in the literature, classifying cyanobacteria into one group is potentially misleading. Cyanobacteria do share a common set of attributes related to cellular organization. However, with respect to phytoplankton community structure, it is more useful to discuss specific cyanobacteria groups. At minimum, it is useful to divide cyanobacteria on the basis of the method used to obtain nitrogen, i.e., heterocystous species that can fix atmospheric $N_2$ and non-heterocystous species that assimilate dissolved inorganic nitrogen. Cyanobacteria-dominated communities have been divided into five "associations", including 1) colonial aggregations of *Anabaena* spp. and *Aphanizomenon* spp., 2) colonial Chroococcales with small cells, 3) *Microcystis aeruginosa*, 4) the filamentous Oscillatoriales of turbid, well-mixed environments, and 5) Oscillatoriales that form metalimnetic layers in relatively deep, stratified lakes (*2*).

The phytoplankton communities of hypertrophic aquaculture ponds are dominated by cyanobacteria in associations 1), 3), and 4). In northwest Mississippi, the most commonly encountered species in commercial catfish ponds are *Oscillatoria agardhii*, *Raphidiopsis brookii*, and *Microcystis aeruginosa* (*3, 4*). Although *Anabaena* spp. occur in catfish ponds in northwest Mississippi, they are not common (*5*). In contrast, species of *Anabaena* and *Microcystis aeruginosa* are common in phytoplankton communities of fish ponds near Auburn, Alabama (*3, 4*). Phytoplankton communities in carp ponds in the Czech Republic are similar to catfish ponds in northwest Mississippi in that communities are dominated by "single-filament" species, such as *Oscillatoria agardhii*, *Oscillatoria redekei*, *Aphanizomenon gracile*, or "single-cell" forms, such as *Microcystis ichtyoblabe* (*6*). Species of *Oscillatoria*, *Spirulina*, and *Microcystis* are common in the phytoplankton communities of brackishwater Israeli aquaculture ponds (*7*).

In catfish ponds in northwest Mississippi, most off-flavors in cultured catfish are caused by MIB, which has been linked to the presence of *Oscillatoria perornata* in the phytoplankton community (*3, 8, 9*). Off-flavors caused by geosmin are much less common than off-flavors caused by MIB in commercial catfish ponds. Geosmin is more common in commercial catfish ponds in Alabama, where it has been associated with *Anabaena* spp. in phytoplankton communities (*3, 10*).

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## Factors Contributing to Dominance of Phytoplankton Communities of Aquaculture Ponds by Cyanobacteria

The composition of phytoplankton communities in aquatic systems results from the interaction between growth-limiting resources and species-specific physiological attributes that confer selective advantage to individual species. Physical, chemical, and biological factors create selective pressures that favor phytoplankton groups that can overcome the limitations imposed by the environment. The predominance of cyanobacteria in the phytoplankton communities of culture ponds can be explained by physiological responses to the selective pressures associated with the supply of limiting resources–primarily nutrient concentration and light intensity–that are exerted on the community. The response to these selective pressures is species-specific. Under the conditions that prevail in shallow, hypertrophic, warmwater aquaculture ponds, a broad array of physiological attributes of cyanobacteria confers selective advantage over other species in the phytoplankton community. Uniquely, these attributes allow them to proliferate and dominate the phytoplankton communities of hypertrophic aquaculture ponds.

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### Water Temperature

Annual variation of water temperature in commercial catfish ponds reflects the prevailing climate of the region. Water temperatures in northwest Mississipii are >20 °C for about 200 d and >25 °C for about 140 d in a year (*11*). Water temperatures that are conducive for good catfish production are also favorable for cyanobacteria growth. Optimum growth of most cyanobacteria occurs at water temperatures >25 °C (*12*). The optimum temperature for growth of *Oscillatoria agardhii* is 32 °C with growth at 20 °C and 36 °C about 60% of that at 32 °C (*13*). Using indirect, statistical evidence, water temperature was estimated to be more important than other physical or chemical factors in controlling *Oscillatoria* density in Lake Okeechobee, Florida (*14*).

Past observations of annual variation in phytoplankton community composition in channel catfish ponds indicated predictable successional patterns in which cyanobacteria were dominant only during the warm summer months. Anecdotal evidence suggests that cyanobacteria (primarily *Oscillatoria agardhii*) blooms in commercial catfish ponds are now much more persistent through the year than in the past. This persistence can be explained in part by the common management practice of not draining ponds between fish crops and the wide temperature tolerance of *Oscillatoria agardhii*. Compared to other bloom-forming cyanobacteria, *Oscillatoria agardhii* tolerates a wider range of temperatures (*12*). In some shallow Dutch lakes, *Oscillatoria* blooms will persist in years with mild winters (*15*). In culture, *Oscillatoria agardhii* does not grow at 5 °C, although the culture remains viable (*16*). Thus, dormant vegetative filaments may occupy the water column during winter, poised to resume active growth once water temperature increases in the spring.

## Underwater Light Climate

The steep attenuation of light as a function of depth is a common feature of all hypertrophic waters, including aquaculture ponds. Phytoplankton growth is intentionally encouraged in fertilized ponds in which growth of cultured fish is based on primary production. In semi-intensive aquaculture ponds in which fish are fed, dense phytoplankton blooms develop unintentionally. The majority of nutrients added to ponds in the form of feed are released to the pond environment. These nutrients stimulate the development of large standing crops of phytoplankton and are supplied far in excess of requirements for phytoplankton growth.

Light plays an indirect role in the development of cyanobacteria dominance of the phytoplankton communities of aquaculture ponds and shallow lakes. Nutrient loading stimulates plant growth generally, initially favoring eukaryotic algal taxa with greater growth rates than cyanobacteria. Cyanobacteria populations develop more slowly, but are increasingly favored as phytoplankton growth makes the water column progressively more turbid. Furthermore, at a comparable nutrient level, cyanobacteria will cause greater turbidity than eukaryotic green algae (*17*), meaning that cyanobacteria consume more light per unit biomass than green algae. Also, cyanobacteria are more susceptible to photo-inhibition at low turbidity and are thus less likely to dominate in relatively clear water.

In fertilized or fed aquaculture ponds, light is severely attenuated with depth and the optical depth of aquaculture ponds (0.2-0.6 m) is often much less than the physical depth (1.0-1.5 m). Phytoplankton growth in hypertrophic aquaculture ponds is considered to be light-limited (*11, 18, 19*). If light does not limit growth, most other groups of phytoplankton grow faster than cyanobacteria. Therefore, cyanobacteria domination of the phytoplankton communities of ponds and lakes can be considered to occur during late succession, as the community approaches the climax or carrying capacity of maximum phytoplankton density.

Cyanobacteria are generally adapted to grow well at lower light intensities than other phytoplankton. For example, the half-saturation light intensity for *Oscillatoria agardhii* is on the order of 4-5 $\mu E\ m^{-2}\ s^{-1}$ and saturation light intensity is reached at 40-175 $\mu E\ m^{-2}\ s^{-1}$ (*13*). *Oscillatoria* are a "shade-adapted" group with relatively low maximum growth rate compared to eukaryotic algae, but with a half-saturation irradiance that is much lower than most eukaryotic algae and many other

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cyanobacteria (*20*). *Oscillatoria* spp. have a light conversion efficiency that is among the most efficient of any phytoplankton group (*21*). The initial slope ($\alpha$) of growth rate as a function of light intensity is an index of light utilization efficiency. Expressing $\alpha$ as a function of the product of surface area-to-volume and the maximum axial length of a cell indicates that the light utilization efficiency of *Oscillatoria agardhii* is much greater than most other phytoplankton (*21*).

Cyanobacteria also contain accessory pigments called phycobilisomes that allow harvesting of energy contained in light frequencies that are characteristic of dimly-lit waters and that are not accessible to plants containing chlorophyll as the primary light-harvesting pigment. The primary accessory pigments of cyanobacteria are the phycobiliproteins, consisting of phycocyanins and phycoerythrins, which complement the light harvesting capability of chlorophylls. *Oscillatoria agardhii* cultured under low light intensity will adapt to the light regime by increasing chlorophyll and c-phycocyanin concentrations (*16*). The ratio of c-phycocyanin to chlorophyll increases as light intensity decreases, indicating a proportionally greater increase of accessory pigment as light intensity decreases.

The phytoplankton communities of many shallow lakes in the Netherlands (*17, 22*), Denmark (*23*), and Germany (*24*) are dominated by filamentous, non-heterocystous cyanobacteria, principally *Oscillatoria agardhii* and *Microcystis aeruginosa*. The Dutch "*Oscillatoria* lakes" (Veluwemeer, Wolderwijd, Drontemeer) are shallow, nutrient rich, and well-mixed from constant exposure to winds from the North Sea. *Oscillatoria* lakes are shallow ($z_{avg}$ <3 m) and have a ratio of Secchi disk visibility to $z_{avg} < 0.3$. There is a strong relationship between light availability (indexed as the product of the light extinction coefficient and lake depth, or *EZ*) and *Oscillatoria* abundance (*17*). Statistical analysis of data from 55 shallow Dutch lakes indicated that *Oscillatoria* will comprise >50% of algal biovolume at a shade (*EZ*) level of about 8, which corresponds to a ratio of optical depth to mixed-layer depth ($z_{eu}/z_m$) of about 0.6 (*17*).

In shallow Dutch lakes, *Oscillatoria* abundance is bi-modally distributed–it is either a major or a minor component of the phytoplankton community (*17*)–suggesting a threshold response to resource availability. This threshold response is exemplified by the results of lake restoration projects in the Netherlands. In Lakes Veluwemeer and Schlachtensee, the abundance of *Oscillatoria* declined abruptly as $z_{eu}/z_m$ increased above 0.4. As a point of reference, the optical depth of 84 small, shallow (1.5 m deep) aquaculture research ponds in Mississippi was 66 cm, or a $z_{eu}/z_m$ of 0.44 (unpublished data).

Although shallow ponds stratify during calm, sunny days, designated as circadiomictic (*21*), stratification is not persistent and the mixed depth can be assumed to be equivalent to the physical depth of the ponds. Therefore, hypertrophic aquaculture ponds have a $z_{eu}/z_m$ that is very similar to the threshold described for shallow Dutch lakes (*22*). Analysis of the data set of 55 shallow Dutch lakes concluded that *Oscillatoria* dominance was one possible equilibrium condition of

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algal community composition, one favored by their shade tolerance, but that other equilibrium conditions were possible if light and nutrient conditions surpassed critical threshold values (*17*). This supposition is reinforced by the finding that chlorophytes supersede cyanobacteria at very high nutrient concentrations (Total Phosphorus >1000 μg/L) (*23*).

## Water Column Stability

Shallow lakes and aquaculture ponds are readily mixed by wind. Wind-induced mixing counteracts the stabilizing force of solar radiation, which can result in pronounced thermal stratification and density gradients between surface and bottom waters during the warm months. Wind-induced water turbulence varies seasonally, with generally low levels of turbulence during summer.

Water movements in shallow lakes and aquaculture ponds are complex. As the extent of mixing increases, phytoplankton cells are circulated between photic and aphotic zones with increasing frequency. Thus, phytoplankton cells experience an oscillating light regime. In shallow, well-mixed ponds and lakes, the frequency of such oscillations is greater than in deeper lakes. Furthermore, phytoplankton may experience extremes of irradiance, ranging from high, potentially photo-inhibitory intensity to low, growth-limiting intensity. Cyanobacteria are favored at low levels of water turbulence (or high levels of water column stability) because they can compete more effectively for light than most other groups by regulating their position in the water column.

Movement of most phytoplankton in natural waters is directed by naturally occurring turbulent mixing, a force opposing water column stability (i.e., stratification). During summer, the water columns of aquaculture ponds stratify during the day and de-stratify at night. Summer is characterized by a high rate of solar energy input, elevated and fairly stable water temperatures, and generally low wind speed. These factors combine to contribute to the development of a stable and stratified water column during the day. Many cyanobacteria can regulate their vertical position in the water column. Development of a cyanobacteria bloom is favored if the rate of turbulent mixing is less than the flotation rate of the cyanobacteria.

Cyanobacteria regulate buoyancy in the water column by two mechanisms. First, cyanobacteria can produce carbohydrate granules during photosynthesis that provide "ballast". Carbohydrate ballast increases cyanobacteria specific gravity relative to water, causing sinking. As the carbohydrate is consumed in cellular metabolism, the cell becomes lighter relative to water and floats to the surface. The second mechanism is associated with the production of gas vacuoles, which consist of coalesced gas vesicles. The production of gas vacuoles occurs in dimly-lit waters and is fueled by the metabolism of accumulated carbohydrate. Gas vacuolation

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causes cells to float to the surface. Near the surface, photosynthesis leads to carbohydrate (polyglucan) production and a resulting increase in internal cell turgor pressure. Increased turgor pressure causes gas vacuoles to burst, reducing the specific gravity of the cell relative to water. The relative importance of the two mechanisms of buoyancy regulation remains an open question, but the cellular ballast mechanism operates at much shorter time scales than the gas vacuole mechanism. In deeper lakes, larger scale vertical movements are probably governed by gas vacuole formation, over which shorter scale vertical water movements associated with carbohydrate ballast are superimposed, all of which are regulated by the availability of light to drive photosynthesis.

Buoyancy regulation by *Microcystis aeruginosa* is far superior to that of other cyanobacteria (*25*). The flotation rate of *Microcystis aeruginosa* (3110 cm/d) is much greater than that of *Anabaena flos-aquae* (518 cm/d) or *Oscillatoria agardhii* (7 cm/d). The sinking rate of *Microcystis aeruginosa* (1,037 cm/d) is also much greater than that of *Anabaena flos-aquae* (86 cm/d) or *Oscillatoria agardhii* (43 cm/d). *Microcystis aeruginosa* can form concentrated "hyperscums" extending from the surface to 40 cm depth in hypertrophic waters (*26*). Such hyperscums have been observed in commercial catfish ponds, particularly along leeward pond edges. The preconditions for hyperscum formation include: 1) large numbers of buoyant cyanobacteria, 2) high irradiance, 3) protracted periods of low wind speed, and 4) a protected site for colony accumulation (*27, 28*).

Competition experiments and mathematical modeling have been used to describe the outcome of competition between phytoplankton species for light (*29, 30, 31*). The model considers the relationship between irradiance and growth, the distribution or availability of light as a function of depth, and turbulent mixing. Each phytoplankton species has a "critical light intensity", defined as the light intensity at the bottom of a mixed water column such that the population size integrated over lake depth does not change. The model predicts that the species with the lowest critical light intensity will become dominant in well-mixed environments. However, in waters that are not well-mixed, regulation of vertical position in the water column becomes more important than critical light intensity as a determinant of the outcome of species competition for light. In lakes and ponds with shallow water columns, low diversity phytoplankton blooms can develop across a wide range of turbulent diffusion (*31*). Model results suggest that the transition from one species to another occurs rather abruptly and that there is only a narrow range of conditions where the species can co-exist.

## Sediment Resuspension

Wind blowing over the surface of shallow lakes and ponds generates surface waves, whose oscillatory motion dissipates exponentially with depth. If the shear

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generated by these water movements at the sediment-water interface exceeds a critical threshold determined by particle density, then sediments will be resuspended into the water column. The frequency and extent of sediment resuspension is inversely related to water depth. Additionally, the foraging activities of fish and some macro-invertebrates can contribute to sediment resuspension. Sediment resuspension is a common occurrence in shallow lakes and aquaculture ponds.

Although sediment resuspension has important implications for nutrient dynamics and underwater light climate, sediment resuspension also affects phytoplankton community dynamics by transporting an inoculum of algal resting stages to the water column. In this way, sediment resuspension functions to provide a "head start" to algal groups that have accumulated resting stages in the sediment. For example, a concentrated layer of plankton (primarily diatoms) accumulates near the sediment surface of Lake Apopka, Florida (*32*). This flocculent layer of meroplankton would be periodically suspended and distributed through the water column when wind speed was sufficiently great; water column chlorophyll concentration was directly correlated with wind speed.

When conditions are unfavorable, many cyanobacteria produce vegetative resting stages (e.g., akinetes) or dormant colonies that settle, accumulate, and remain viable in the sediment. Cyanobacteria trichomes do not decompose and remain viable after three months under ice cover (*33*). Using stable isotope tracers, *Microcystis aeruginosa* has been shown to overwinter on the sediment surface (*34*). Thus, the sediment may serve as a "seed bank" for cyanobacteria colonization of the water column.

When conditions become favorable, activation of resting stages allows rapid development of cyanobacteria populations relative to other species with lower inoculum densities or that are introduced by other dispersal mechanisms. The sediment of shallow lakes and the littoral zones of deeper lakes may be exposed to warm temperatures and sufficient irradiance to cause germination of akinetes in the spring and are subject to a greater frequency and extent of sediment resuspension than deeper lake regions.

The phytoplankton communities of aquaculture ponds often develop into low-diversity communities dominated by a small number of species, primarily cyanobacteria. Additionally, the accumulation of resting stages of cyanobacteria in the sediment of commercial catfish ponds is promoted by the common management practice of not draining ponds between fish crops. Most commercial channel catfish ponds used for the production of food fish are managed as static water systems that are not drained for 10 years or more. Thus, once dominance by cyanobacteria is established, accumulation of resting stages in the sediment will favor cyanobacteria. When conditions are favorable for growth, sediment resuspension will aid in the rapid colonization of the water column and accelerate dominance and promote persistence of cyanobacteria blooms.

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Despite the potential importance of this mechanism as a factor contributing to cyanobacteria dominance of commercial catfish ponds, Head et al. (*35*) suggested that small overwintering pelagic populations of cyanobacteria were more important than benthic recruitment as the primary source of inocula for summer populations of cynaobacteria in mesotrophic Esthwaite Water, UK. However, in this relatively deep (12 m) lake, benthic recruitment was associated with vertical movements of buoyant cyanobacteria and not sediment resuspension.

## Nutrient Concentrations

Numerous attempts have been made to assess the effect of nutrients on phytoplankton community structure. Phytoplankton growth follows Michaelis-Menten enzyme-substrate kinetics and so each species can be described in terms of a maximum growth rate ($\mu_{max}$) and a half-saturation concentration ($K_s$) for a particular nutrient. In general, growth limiting nutrient concentrations rarely prevail in natural waters, and certainly not in semi-intensive aquaculture ponds. Based on the Redfield ratio, Reynolds (*36*) calculated that the minimum concentrations to satisfy requirements for growth of most phytoplankton species are rarely more than 3 µg/L for dissolved P and 100 µg/L for dissolved N. Average soluble reactive P concentrations in commercial channel catfish ponds range from about 25 µg/L during winter to 100 to 150 µg/L during late spring (*37*). Average dissolved inorganic nitrogen concentrations in commercial channel catfish ponds range from 500 µg/L during summer to 3000 µg/L during winter (11). Thus, nutrient flux rates are usually sufficient to maintain concentrations that saturate growth of phytoplankton in aquaculture ponds.

Based on studies of 17 eutrophic lakes worldwide, Smith (*38, 39*) suggested that the occurrence of nuisance blooms of cyanobacteria were correlated with N:P ratios and that low ratios (<25 to 30 by weight) favor dominance by cyanobacteria. The argument is based on the presumed superior competitive ability of heterocystous cyanobacteria to obtain inorganic nitrogen through fixation of atmospheric $N_2$ when N concentrations are low relative to P.

Cyanobacterial nitrogen fixation is restricted to those genera with heterocysts, including *Anabaena* and *Aphanizomenon*. Geosmin produced by some species of *Anabaena* is one of the two principal off-flavor causing compounds in cultured catfish. Paerl (*40*) emphasized the role of cyanobacteria-microbial consortia in maintaining micro-scale anaerobiosis around cyanobacteria *(Anabaena, Aphanizomenon)* heterocysts. Indeed, nitrogen fixation does not occur in axenic cultures of heterocystous cyanobacteria.

Nitrogen fixation by heterocystous cyanobacteria is inducible. Ammonia is the nitrogenous substrate that is preferred by heterocystous cyanobacteria. Only when ammonia and nitrate supplies are depleted will heterocystous cyanobacteria fix di-

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nitrogen. Low inorganic nitrogen concentration may be a selective factor favoring heterocystous cyanobacteria in the epilimnion of stratified lakes and reservoirs, but the same nutrient dynamics do not apply to shallow lakes and aquaculture ponds. First, nutrient loading rates of semi-intensive aquaculture ponds are high. Second, mineralization of organic matter at the sediment-water interface, diffusion of ammonia from the sediment, pond mixing, and sediment resuspension combine to provide continuous or pulsed input of nutrients to the water column. It has not been established that inorganic nitrogen concentrations in hypertrophic aquaculture ponds are sufficiently low to provide selective advantage to heterocystous cyanobacteria.

Furthermore, the importance and relevance of the N:P ratio as a factor affecting phytoplankton community structure and specifically the dominance of phytoplankton communities by cyanobacteria has been called into question (*23, 36, 41, 42*). First, the N:P ratio decreases as total P increases, so the two factors are collinear and the effects can not be separated. A more robust analysis of the lake data used by Smith (*38*) indicated that total P or total N were better predictors of cyanobacteria biomass and cyanobacteria dominance (i.e., cyanobacteria biomass as a proportion of total phytoplankton biomass) than the N:P ratio (*41*).

Downing et al. (*43*) analyzed data from 99 lakes and determined that phytoplankton biomass was the best single explanatory variable of cyanobacteria dominance, followed by single nutrients (N and P). Phytoplankton biomass can be interpreted in this context as an index of light availability because light availability decreases as phytoplankton biomass increases. The N:P ratio was the worst single explanatory variable of cyanobacteria dominance. Similarly, total algal biomass was the best predictor of cyanobacteria biomass in 165 shallow Florida lakes (*42*). At a dry weight phytoplankton density of 50 to 100 mg/L, cyanobacteria represented a wide range of proportion of total phytoplankton biomass, but cyanobacteria were dominant if phytoplankton density was >100 mg/L. At N:P ratios <29, the proportion of total phytoplankton biomass that was cyanobacteria was much more variable than at N:P ratios >29. In other words, some lakes with a low N:P ratio were dominated by cyanobacteria, but many other lakes with a low N:P ratio were dominated by other groups of phytoplankton.

Using data provided by the National Eutrophication Survey conducted by the U.S. Environmental Protection Agency in the mid-1970s, Harris (*44*) estimated the probability of occurrence of nuisance blooms of cyanobacteria in 435 lakes and reservoirs as a function of the N:P ratio and an index of water column stability. The probability of occurrence of *Anabaena* or *Aphanizomenon* blooms increased when N:P ratio was <30. *Microcystis* blooms occurred at a broad range of N:P ratios, but were more common in lakes with stable water columns than in well-mixed lakes. Low N:P ratio was not a good predictor of *Oscillatoria* blooms, which were most prevalent in well-mixed lakes at a fairly broad range of N:P ratios.

Furthermore, Reynolds (*36*) suggested that enzyme-mediated active transport systems are able to "sense" the concentration of individual nutrients, but are not able to perceive nutrient ratios. Individual nutrient concentrations are important, but the ratio between nutrients has no direct physiological relevance. Reynolds (*36*) argues that nutrient ratios are the outcome of nutrient uptake by algae cells, not a determinant or predictor of the relative competitive ability of individual species for nutrients.

Nutrient limitation may be an important factor affecting the transition of phytoplankton communities from chlorophytes to cyanobacteria (*45*). However, nutrient limitation in hypertrophic aquaculture ponds with a continuous supply of nutrients from external sources (e.g., feeding or fertilization) and internal sources (e.g., rapid nutrient recycling) is not likely. Despite elevated demand for nutrients by a large phytoplankton biomass during summer, nutrient concentrations are not likely to be growth-limiting. Nitrogen-fixing species, indicative of nutrient (N) limiting conditions, are not the dominant cyanobacteria in Mississippi catfish ponds.

If N:P ratio was an important determinant of phytoplankton community structure in aquaculture ponds, then heterocystous genera such as *Anabaena* would dominate. However, the phytoplankton communities in most commercial channel catfish ponds are dominated by non-heterocystous genera such as *Oscillatoria* and *Microcystis*. Also, the presence of heterocystous cyanobacteria in aquaculture ponds does not necessarily implicate N:P ratio as a selective factor. Nitrogen fixation by heterocystous cyanobacteria is inducible and can be suppressed by ammonia, which is the inorganic nitrogen substrate preferred by phytoplankton.

In particular, ammonia may be a selective factor that favors non-heterocystous cyanobacteria. Blomqvist et al. (*46*) provide evidence from whole-lake fertilization studies and suggested that cyanobacteria are superior competitors for ammonia than other phytoplankton and that eukaryotic phytoplankton are superior competitors for nitrate. These differences have been attributed to biochemical differences in the pathways of inorganic nitrogen assimilation between cyanobacteria and eukaryotic algae (*45*). In a series of enclosure experiments, Klemer (*47*) demonstrated that *Oscillatoria agardhii* density increased when enclosures were fertilized with ammonia and phosphate, but decreased when enclosures were fertilized with nitrate and phosphate. During the growing season in semi-intensive aquaculture ponds, ammonia is supplied continuously from fish excretion. The flux of ammonia from the sediment is an important source of nitrogen in shallow lakes (*23*) and aquaculture ponds (*48*). Thus, the high affinity for ammonia likely contributes to the dominance of many Mississippi catfish ponds by *Oscillatoria agardhii.*

Hyenstrand et al. (*45*) suggested that cyanobacteria can overcome nutrient limitation in the epilimnion of stratified lakes by vertical migration between the nutrient-rich hypolimnion and the well-lit epilimnion, or by nitrogen fixation. In well-mixed shallow lakes and aquaculture ponds, stratification does not exert as

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strong a selective force on phytoplankton community composition as in deeper, seasonally stratified lakes. In shallow lakes and aquaculture ponds, nutrients may be obtained by sinking to the nutrient-rich sediment-water interface.

Downing et al. (*43*) estimated that the risk of cyanobacteria dominance increases rapidly between total P concentrations between 30 and 70 µg/L and reaches an asymptote at about 100 µg/L. A similar threshold response was observed during the restoration of two Dutch lakes dominated by *Oscillatoria agardhii*. Cyanobacteria dominance declined rapidly at a total P concentration <200 µg/L in Lake Schlactensee and <100 µg/L in Lake Veluwemeer (*22*). As a point of reference, total P concentrations in commercial channel catfish ponds range from 300 to 400 µg/L during the winter and spring increasing to 700 µg/L during the late summer (*37*). Thus, a substantial reduction of nutrient concentrations in catfish ponds would be required before potential changes in phytoplankton community structure could occur.

Jensen et al. (*23*) conducted a correlation analysis of data from a survey of 178 shallow lakes in Denmark. Non-heterocystous cyanobacteria, mainly *Microcystis*, dominated the phytoplankton communities of lakes with TP between 250 and 800 µg/L, similar to the concentrations of TP that prevail in commercial catfish ponds. However, chlorophytes often dominated the phytoplankton communities of lakes where TP was >1000 µg/L. This result was attributed to the competitive superiority of chlorophytes for nutrients when nutrient supply rates, primarily from sediment nutrient flux or resuspension, and nutrient concentrations are high (i.e., saturating). A further prerequisite for chlorophyte dominance at high TP was assumed to be low grazing pressure from zooplankton resulting from the presence of planktivorous fish.

## Carbon Dioxide Concentration and pH

High rates of photosynthesis in hypertrophic aquaculture ponds can result in the depletion of carbon dioxide and the elevation of pH by mid-afternoon. Cyanobacteria dominate the phytoplankton communities of wastewater treatment ponds when carbon dioxide concentration is low and pH is high (*49*). Hypotheses advanced to explain cyanobacteria dominance under such conditions have attempted to explain the observed pattern as an outcome of the greater affinity for carbon dioxide by cyanobacteria compared to eukaryotic algae, active transport of carbon dioxide or bicarbonate by cyanobacteria, or differential effects of pH on inorganic carbon or other nutrient uptake.

However, pH is proportional to the ratio between carbon dioxide and alkalinity and so differentiation of the effects of pH and carbon dioxide on inorganic carbon uptake must consider alkalinity. Caraco and Miller (*50*) were able to distinguish between the effects of pH and carbon dioxide on competitive outcomes between

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a cyanobacterium (*Aphanizomenon flos-aquae*) and chlorophytes (*Scendesmus* spp. and *Selenastrum* spp.). They concluded that both pH and carbon dioxide had an effect on cyanobacteria dominance, but that pH had a greater effect than carbon dioxide, with cyanobacteria representing an increasing proportion of the algal community as pH increased. At pH >8.5, variation in carbon dioxide concentration did not have a large effect on cyanobacteria abundance. However, cyanobacteria abundance increased with decreasing carbon dioxide at pH 7. Caraco and Miller (*50*) proposed several mechanism by which high pH could confer advantage to cyanobacteria, including pH effects on nutrient uptake enzymes, differential uptake of different ortho-phosphate forms, and reduced metal solubility.

One hypothesis that has been advanced to explain the dominance of cyanobacteria in lakes with low carbon dioxide concentration or high pH holds that cyanobacteria have a higher affinity for carbon dioxide than eukaryotic algae. Caraco and Miller (*50*) developed a model that compared favorably with the results of their competition experiments. In the model, the half-saturation concentration ($K_m$) for carbon dioxide uptake was 1 μM for cyanobacteria and 15 μM for eukaryotic algae. However, evidence for a greater affinity for $CO_2$ by cyanobacteria than eukaryotic algae is equivocal. In common with eukaryotic algae, cyanobacteria use the ribulose bisphosphate carboxylase/oxygenase (Rubisco) enzyme for carbon dioxide fixation. As Rubisco has a relatively low affinity for $CO_2$, presence of this enzyme does not confer any selective advantage to cyanobacteria with respect to aqueous $CO_2$ concentration. However, cyanobacteria can increase intracellular $CO_2$ concentration around the Rubisco enzyme through active transport of $CO_2$ and $HCO_3^-$. Active transport of $HCO_3^-$ is followed by dehydration to $CO_2$ by carbonic anhydrase (*51*). Active transport of $CO_2$ and $HCO_3^-$ has been demonstrated in *Synechococcus*, *Coccochloris*, *Spirulina*, and *Anabaena variabilis*, but it is not clear whether such active transport mechanisms are conserved across other genera of bloom-forming cyanobacteria.

Shapiro (*52*) suggested that low carbon dioxide or high pH does not *cause* cyanobacteria to become dominant in the phytoplankton. For example, one basin of Squaw Lake, Wisconsin was destratified and injected with carbon dioxide and one basin was not treated. The timing and density of a nuisance bloom of *Aphanizomenon flos-aquae* and *Anabaena flos-aquae* was not affected, despite large differences in pH and carbon dioxide concentrations. Shapiro (*52*) speculated that, once cyanobacteria become dominant, usually as a major component of high-density algal communities, they create the environmental conditions such that other algal species are unable to compete effectively for low carbon dioxide concentration.

Most well-buffered freshwaters contain abundant bicarbonate. Mass development of cyanobacteria has been attributed in part to the presumed ability of some cyanobacteria (e.g., *Microcystis aeruginosa*) to utilize bicarbonate directly, although the evidence for such a mechanism (*53*) is indirect and equivocal. Shapiro

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(*52*) was unable to demonstrate direct fixation of bicarbonate and dismissed the mechanism as an explanation for cyanobacteria dominance of algal communities. Moreover, algal affinity and preference for carbon dioxide is much greater than that for bicarbonate. To illustrate this point, the $K_m$ was 1 μM for carbon dioxide and 200 μM for bicarbonate uptake by cyanobacteria in the model developed by Caraco and Miller (*50*).

The importance of low carbon dioxide concentration as a factor affecting algal community composition in semi-intensive aquaculture ponds is not known. However, in common with other shallow and generally well-mixed aquatic systems, carbon dioxide may not be of great importance as a determinant of phytoplankton community composition because the supply of carbon dioxide to the photic zone of shallow lakes is likely greater than that to the photic zone of deeper lakes (*23*). Two sources of carbon dioxide are of much greater importance in shallow, weakly stratified lakes and aquaculture ponds than in the deeper, seasonally stratified lakes where much of the research related to the effect of carbon dioxide and pH on phytoplankton community composition has been conducted (e.g., *52*). First, rapid mineralization of easily decomposed organic matter at the sediment-water interface supplies large amounts of carbon dioxide to the overlying water. Second, atmospheric diffusion of carbon dioxide across the air-water interface is likely to be much more important as a source of carbon dioxide in shallow, well-mixed lakes than in deep, stratified lakes. Nonetheless, low carbon dioxide concentrations and high pH can prevail for several hours each day during the growing season, even in well-buffered water. The extent to which this relatively brief period confers advantage to cyanobacteria, thereby affecting algal community composition, is unclear.

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## Trace Metal Availability

At the alkaline pH values that normally prevail in hypertrophic aquaculture ponds, trace metals are poorly available. Furthermore, organo-metallic complexes and precipitation of metal hydroxides reduce trace metal availability. Trace metals are important components of carbon and nitrogen metabolism in cyanobacteria. Iron is involved in electron transport and phycobilin biosynthesis associated with photosynthesis, and both iron and molybdenum are involved in nitrogen metabolism. Of the many trace metals required for phytoplankton growth, iron is most likely to potentially limit growth of cyanobacteria. The argument is based on the requirement for iron in nitrogenase and reductase enzymes involved in nitrogen fixation and nitrate reduction (*54*). Thus, heterocystous cyanobacteria are likely to be most sensitive to iron limitation.

Some cyanobacteria are able to secrete siderophores–extracellular, low molecular weight chelators–that can bind to metals and carry them to the cell surface where they are transported into the cell. Siderophore production represents a high-affinity transport system for trace metals. Siderophore production has been demonstrated in coccoid (*Synechococcus*, *Synechocystis*) and filamentous (*Oscillatoria tenuis*, *Anabaena catenula*) cyanobacteria (*55*). Although cyanobacteria release siderophores to the environment, there is no evidence that siderophore-complexed metals are used exclusively by cyanobacteria. Thus, the selective advantage of siderophore production as an ecophysiological attribute favoring cyanobacteria is an open question.

Furthermore, siderophore production is most likely to occur under nitrogen limitation, a condition that rarely prevails in hypertrophic aquaculture ponds that receive regular nutrient loading. Additionally, commercial catfish ponds in northwest Mississippi are supplied with groundwater of high iron content, so large quantities of iron are added when ponds are filled. Although this iron may precipitate to the sediment as ferric hydroxides or oxyhydroxides, and therefore not be biologically available, periods of low dissolved oxygen concentration can lead to transient sediment anoxia, ferric-iron reduction, and the pulsed release of available iron to the water column.

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## Resistance to Zooplankton Grazing

Cyanobacteria vary greatly in size, ranging from picoplanktonic forms to large, filamentous or colonial, coccoid forms. In general, larger cyanobacteria are resistant to grazing by filter-feeding zooplankton. Resistance to grazing has been attributed to the large size of cyanobacteria that causes clogging of feeding apparatus, particularly of larger cladoceran zooplankton (e.g., *Daphnia* spp.).

Copepods are generally more effective grazers of cyanobacteria than cladocerans because they employ both raptorial capture of large particles as well as passive filtration of small particles (*56*). Copepods can also discriminate among different cyanobacteria. However, the selectivity of *Oscillatoria agardhii* by the copepod *Diaptomus birgei* was among the lowest of 16 cyanobacteria taxa tested (*56*).

Furthermore, cyanobacteria may produce toxins that render cyanobacteria filaments or colonies unpalatable or toxic when consumed. Toxic cyanobacteria may be more toxic to zooplankton than to vertebrates (*56, 57*). Feeding rates of *Daphnia* fed toxic strains of *Microcystis* are lower than those fed non-toxic strains (*57*). Large-bodied zooplankton, such as *Daphnia*, are more susceptible to cyanobacteria toxins because they are more likely to ingest large filamentous or colonial cyanobacteria than small-bodied zooplankton (*58*).

Resistance to grazing varies with zooplankton group. Although large-bodied cladocerans are more likely to ingest large cyanobacteria, they have particular difficulty doing so. Large filamentous or colonial cyanobacteria are simply too large for small-bodied zooplankton. Thus, cyanobacteria blooms can affect zooplankton community structure by favoring small-bodied forms (e.g., rotifers) that can graze upon nanoplankton sub-dominant in a cyanobacterial bloom.

### Allelopathy

Of the inorganic carbon fixed by phytoplankton, some fraction is incorporated into cells to form new biomass, but a large fraction is released to the environment as dissolved organic matter. Some of these organic compounds have allelopathic effects. Allelopathy describes the effect of compounds released by plants that (usually negatively) affects the growth of other plants. Some cyanobacteria produce compounds that may have a negative effect on the growth of potentially competing algae. For example, species of *Anabaena* release heat-labile compounds that can suppress diatom growth (*59, 60*).

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## Opportunities for Manipulation of Phytoplankton Communities

The opportunities to manipulation the phytoplankton communities of aquaculture ponds to shift dominance away from cyanobacteria are extremely limited. First, reducing nutrient supply is not practical because fish must be fed. The inherent inefficiency of nutrient utilization in aquaculture ponds means that most nutrients are released to the pond environment and so fish culturists must understand the consequences of nutrient enrichment and manage accordingly. One of these consequences is dense phytoplankton blooms dominated by cyanobacteria. The available management options are constrained by practical or economic considerations. The foregoing discussion will focus on those management practices that are related to cyanobacteria ecophysiology, with particular emphasis on addressing resource limitation. Management of cyanobacteria blooms with chemicals is addressed elsewhere in this volume.

### Mixing

Hypertrophic aquaculture ponds are very turbid, thereby restricting the availability of light energy for photosynthesis. These conditions select for shade-adapted species of phytoplankton that are able to harvest light energy at low irradiance intensities. Opportunities for reducing nutrient supply are limited in the context of aquaculture ponds that routinely are provided with large quantities of nutrients in the form of feeds and fertilizers. The regular addition of nutrients in the form of feeds is required for economical, semi-intensive pond aquaculture. Thus, methods to provide more light to the water column of hypertrophic aquaculture ponds presents an opportunity to manipulate phytoplankton community composition. One obvious way to improve the underwater light climate in hypertrophic ponds is to increase water turbulence through mechanical mixing. Mixing can increase the "average" exposure of phytoplankton cells to light, as cells are circulated between light-saturating and light-limited zones of the water column.

Research on mixing aquaculture ponds has justifiably focused on assessments of water quality and fish production and has not explicitly assessed phytoplankton community structure or the incidence of off-flavor. Research conducted at Clemson University has evaluated a novel approach to mixing ponds. The partitioned aquaculture system (PAS) consists of fish culture raceways hydraulically connected to a shallow high-rate algal pond by a paddlewheel rotating at 2-3 rpm to provide water movement. Thus, turbulent mixing in the PAS is far greater than in conventional aquaculture ponds.

Analysis of the phytoplankton community structure in the PAS provides some of the most compelling evidence that mixing of aquaculture ponds is insufficient to shift phytoplankton communities in hypertrophic aquaculture ponds from cyanobacteria dominance. However, phytoplankton communities in the PAS are dominated by *Microcystis* (*61*) not *Oscillatoria agardhii*, the dominant cyanobacteria in commercial catfish ponds in northwest Mississippi. *Microcystis* is a cyanobacteria taxon that has not been implicated in episodes of off-flavor in commercial catfish aquaculture. Although commercial catfish ponds and the PAS receive abundant nutrients derived from feeding fish, a mechanistic explanation of why *Microcystis* rather than *Oscillatoria* dominates the phytoplankton communities of the PAS is lacking.

Dense populations of *Microcystis* often form surface scums and are therefore exposed to levels of irradiance that can lead to photo-oxidative death and stress. In response to exposure to such high levels of irradiance, particularly in the ultra-violet and near ultra-violet wavelengths, *Microcystis*, *Anabaena*, and *Aphanizomenon* can synthesize carotenoid pigments (xanthophylls and β-carotene) that protect these cyanobacteria from photo-oxidation (*62, 63*). These carotenoids function as a protective "sun-screen" for cyanobacteria that are repeatedly exposed to potentially photo-inhibitory irradiance in well-mixed ponds.

Further evidence for the selective advantage of *Microcystis* over *Oscillatoria* in the PAS can be derived from analysis of the relationship between growth and irradiance, particularly the (high) irradiance level that causes growth reduction. A photon flux density >200 μE $m^{-2}$ $d^{-1}$ causes photo-inhibition of *Oscillatoria agardhii* (*13*) whereas photo-inhibition occurred at >1000 μE $m^{-2}$ $d^{-1}$ in laboratory cultures and >2500 μE $m^{-2}$ $d^{-1}$ in natural populations of *Microcystis aeruginosa*

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(*64*). Mid-summer surface photosynthetically active radiation in the southeast United States is about 2,000 μE $m^{-2}$ $d^{-1}$. Thus, *Oscillatoria*, a supremely shade-adapted plant, may be exposed to photo-inhibitory irradiance in the well-mixed PAS, whereas *Microcystis* is able to tolerate and indeed thrive in such a nutrient-rich, relatively high-irradiance environment.

Despite the dominance of shallow, well-mixed lakes by *Oscillatoria agardhii*, the experience of the PAS suggests that turbulence greater than that provided by wind mixing can offset the high light utilization efficiency of *Oscillatoria agardhii*, resulting in a shift in community composition to species (albeit primarily cyanobacteria) with greater growth rates, light requirements, or tolerance of high irradiance.

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## Phosphorus Inactivation

The work of Smith (*38, 39*) indicating that nitrogen-fixing cyanobacteria were favored by low N:P ratio raised the possibility of manipulating this variable as a means to control phytoplankton community structure. Increasing the N:P ratio can be accomplished by increasing nitrogen relative to phosphorus or reducing phosphorus relative to nitrogen. Attempts to shift dominance of phytoplankton communities in eutrophic lakes and ponds by cyanobacteria through addition of nitrogen have not been successful (*65, 66*). In hypertrophic aquaculture ponds that receive regular inputs of nutrients, reducing phosphorus relative to nitrogen is a more practical approach. The practicality of phosphorus reduction is further supported by research indicating that total P or N were better predictors of cyanobacteria biomass or dominance than N:P ratio and that total phosphorus was inversely correlated with N:P ratio. Phosphorus has been chemically precipitated from catfish pond water with aluminum sulfate (*67*) and calcium sulfate (*68*). However, this research did not evaluate the effects of such applications on phytoplankton community structure. Furthermore, the effects of alum and gypsum application have no residual effect. Phosphorus derived from additions of feed or fertilizer that follow alum or gypsum application will not be removed. Thus, nutrient forcing of increased phytoplankton biomass and cyanobacteria dominance of phytoplankton communities of hypertrophic aquaculture ponds are not changed by occasional additions of phosphorus-precipitating chemicals.

## Mechanical Algal Harvesting

Large standing crops of phytoplankton are a conspicuous feature of hypertrophic aquaculture ponds. In principle, harvesting primary production would reduce algal density and therefore relieve some of the resource constraints, particularly light availability, that favor cyanobacteria over other phytoplankton groups. Furthermore, filamentous cyanobacteria are large and slow-growing and therefore should be susceptible to mechanical removal. In practice, harvesting phytoplankton is constrained by logistical and economic concerns.

Harvesting algal biomass has been a major technological hurdle in commercial algae production. Commercial algae production methods employ a range of harvesting technologies including gravity filtration, microstrainers, lamella separators, belt filters, vacuum drum filters, chamber filter presses, flocculation, dissolved air flotation, and centrifugation (*69*). Although these technologies could certainly be applied to harvesting algae from aquaculture ponds, current pond management systems and economic considerations will likely limit their application.

Cyanobacteria can be more difficult to harvest than other phytoplankton taxa, thereby restricting the technological options. Gas vacuolate groups tend to float, so removal by sedimentation would be difficult. Other groups produce polysaccharide coatings that would clog the mesh of belt or drum filters, quickly reducing harvest efficiency.

Despite the constraints, aquaculturists and engineers continue to express interest in mechanical harvesting of algae from aquaculture ponds. A prototype algae harvesting unit based on additions of iron salts to enhance flocculation and settling of algae has been installed in the PAS (*70*). Limited operation of the unit has provided some encouraging results, warranting further evaluation. However, the installation of the PAS algal settling unit on a conventional catfish pond would likely interfere with normal pond management and result in lower algal removal efficiency than that obtained in the PAS.

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## Biomanipulation

So-called "top-down" approaches to controlling phytoplankton biomass are based on effects that cascade from the highest trophic levels to the plant base. The key to successful reduction of phytoplankton biomass is increasing the density of large-bodied zooplankton, particularly cladocerans. Therefore, biomanipulation typically focuses on removing planktivorous fish by stocking carnivorous fish. With respect to cyanobacteria blooms, successful biomanipulation presents additional difficulties. The lack of success of biomanipulation as a means to reduce cyanobacteria blooms can be explained by three reasons: 1) zooplankton feeding, growth, and reproduction on a diet of filamentous or colonial cyanobacteria is poor, 2) toxic strains of cyanobacteria are not consumed as readily as non-toxic strains, and 3) cyanobacteria cause a shift in zooplankton community structure to small-bodied forms (e.g., rotifers) that have low grazing rates of cyanobacteria (*71*).

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Although most efforts at biomanipulation have focused on cascading trophic effects, with particular emphasis on zooplankton grazing of phytoplankton, some attempts at top-down control of phytoplankton community structure have focused on stocking fish with finely spaced gill rakers or other mechanisms by which phytoplankton can be removed from water directly by fish. In general, stocking planktivorous fish does not cause a reduction in phytoplankton biomass (*18, 72*). However, filter-feeding fish can have a profound effect on phytoplankton community structure by grazing large filamentous and colonial cyanobacteria. The effect of filter-feeding fish on phytoplankton biomass and community structure depends in part on stocking density. In an extreme example, the phytoplankton community structure was dominated by picoplankton and nanoplankton <10 μm in Chinese carp polyculture ponds stocked with a high density of silver carp (*73, 74*). Thus, although the effects of incorporating filter-feeding fish on overall phytoplankton biomass are equivocal, the positive effects on phytoplankton community structure are more definitive.

## Microbial Pathogens

Development of biological control of cyanobacteria has not yet reached the stage of large-scale experimentation, but has great potential. A wide range of micro-organisms hold promise as biological control agents, including viruses, bacteria, fungi, actinomycetes, and protozoa. This diverse group of biological control agents has a comparably diverse mode of action, ranging from promotion of cell lysis following infection to direct consumption (grazing). In many cases, pathogens are specific to a particular host. Compared to the state-of-knowledge of host-pathogen relationships in terrestrial plants, research on microbial pathogens of aquatic plants is in an early stage of development.

The density of viruses in lakes is very high ($10^5$-$10^8$/ml) (*75*) and is usually one order of magnitude greater than bacterial density (*76*). Viral density has been additionally correlated with chlorophyll *a* and total phosphorus concentrations (*76*). The existence of cyanophages, viruses that infect cyanobacteria, has been known for nearly 40 years (*77*). Cyanophages infect cyanobacteria and cause cell lysis. Direct contact between phage and cyanobacteria is required for infection. Unlike bacteriophages, cyanophages require light for absorption to cyanobacteria and cyanophage replication depends on photosynthetic activity of cyanobacteria (*78*). Cyanophage actvity is favored by low pH. Shapiro (*79*) suggested that increased whole-lake $CO_2$ concentration and reduced pH following lake circulation stimulated cyanophage production.

Cyanophages appear to be specific to particular cyanobacteria hosts. Phlips et al. (*80*) have identified cyanophages in Florida lakes that kill *Anabaena circinalis*, *Anabaena flos-aquae*, *Microcystis aeruginosa*, and *Lyngbya birgei*. Manage et al.

(81) described a cyanophage infection of a *Microcystis aeruginosa* bloom and suggested that the cyanophage was specific to the *Microcystis aeruginosa* host.

The role of cyanophages in the dynamics of cyanobacteria blooms is not clear. Desjardins (*78*) suggested that cyanophages may be more effective in the prevention of cyanobacteria blooms than in controlling the bloom once it has become established. The role of cyanophages in bloom collapse is similarly unclear. Cyanophage infection can initiate bloom collapse (*82*) or can accelerate collapse once it has started.

Algal-lysing myxobacteria and cyanobacteria co-exist in eutrophic waters and densities are closely related. Gram-negative myxobacteria that lyse cyanobacteria have been isolated and identified (*82, 83*). Unlike cyanophages, myxobacteria do not have host specificity, as they are able to lyse phytoplankton, filamentous cyanobacteria, and some eubacteria. Myxobacteria can lyse *Oscillatoria*, *Microcystis*, *Anabaena*, *Aphanizomenon*, and *Gleotrichia* (*83*). A high myxobacteria density ($>10^6$/ml) is required to achieve control of cyanobacteria density (*84*), thereby limiting the potential application of this organism in cyanobacteria control programs. However, using myxobacteria in combination with actinomycetes was more efficient at reducing *Aphanizomenon flos-aquae* density than either pathogen alone (*85*), raising the possibility of improving the effectiveness of cyanobacteria control with myxobacteria using a combined pathogen approach. Despite the low host specificity of myxobacteria, a bacterial pathogen specific for *Oscillatoria* spp. and *Anabaena* spp. has been isolated (*86*). The bacterium does not affect the densities of various chlorophytes or *Microcystis aeruginosa*.

Grazing by the ciliate *Nassula* has effectively reduced the density of blooms of *Oscillatoria agardhii* (*87*), *Anabaena*, and *Aphanizomenon* (*88*). Predation of *Nassula* by cylcopoid copepods and juvenile planktivorous fish (*Rutilus rutilus*) can suppress the effectiveness of cyanobacteria grazing by *Nassula* (*87*). Thus, grazing by ciliates or other protozoans is more effective in the absence of predation by zooplankton or planktivorous fish.

Full-scale application of cyanophages, mxyobacteria, actinomycetes, or protozoans as a means to control cyanobacteria in aquaculture ponds has not yet been attempted. The practicality of inoculating aquaculture ponds with these organisms is a questionable approach. Manipulation of the physico-chemical environment to establish conditions that promote the abundance or activity of these biological control organisms may be more fruitful and cost-effective.

## Conclusion

Worldwide, cyanobacteria proliferate and dominate the algal communities in a wide range of extreme environments, including thermal springs and polar ice.

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Semi-intensive aquaculture ponds are an extreme environment insofar as the availability of light is the primary factor regulating algal community composition. Filamentous cyanobacteria, particularly those in the genus *Oscillatoria* and *Anabaena*, possess physiological adaptations that allow them to flourish in the dimly lit waters of semi-intensive aquaculture ponds. From an ecological standpoint, control methods that consider cyanobacteria physiological attributes and responses to limiting factors have a good probability for success. However, current pond configurations, fish production practices, and water quantity and quality management techniques impose severe constraints on approaches to manipulate phytoplankton community composition based on current knowledge of cyanobacteria ecophysiology.

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## Chapter 10

# Copper Sulfate to Manage Cyanobacterial Off-Flavors in Pond-Raised Channel Catfish

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Pond-raised channel catfish (*Ictalurus punctatus*) often develop off-flavors caused by earthy-musty cyanobacterial metabolites. Management of off-flavors in catfish aquaculture is difficult and algicides are the only management tool that yields relatively dependable results. This chapter reviews the use of copper sulfate to manage off-flavors in catfish from ponds in northwest Mississippi. Weekly treatments with 0.12 mg/L Cu during the summer reduces off-flavor prevalence and improves economic performance. However, copper accumulates in the sediments of treated ponds, with a relatively large fraction of the sediment copper initially present in potentially bioavailable forms.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

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Farm-raised channel catfish (*Ictalurus punctatus*) have gained a large share of the seafood market in the United States. The popularity of catfish is due, in part, to its consistent, mild flavor. Oddly, these favorable attributes belie the fact that many catfish develop highly undesirable "off-flavors" prior to harvest. For the most part, consumers are unaware that pre-harvest off-flavors are a widespread problem because processors screen fish for flavor quality before harvest. If a sample of fish from a pond scheduled for harvest is determined to be off-flavor, fish in that pond will not be accepted for processing until flavor quality improves. Although pre-harvest flavor screening reduces the impact of inconsistent flavor quality at the market level, the inability to harvest and sell off-flavored fish is a serious economic burden for farmers because flavor problems may prevent the timely harvest of fish, increase production costs, reduce revenues, disrupt cash flows, and interrupt the orderly flow of fish from farm to processor (*1*).

Most off-flavors in pond-raised fish are caused by odorous compounds synthesized by cyanobacteria (*2*). Although various approaches have been proposed to manage off-flavors in commercial aquaculture, most attempts have either been unsuccessful or too costly. This chapter reviews one approach to management of cyanobacterial off-flavors—using the algicide copper sulfate ($CuSO_4 \cdot 5H_2O$) to eliminate odor-producing cyanobacteria. We also present information on the accumulation, potential bioavailability, and toxicity of copper in sediments of catfish ponds receiving routine applications of copper sulfate.

## Management of Off-flavors in Catfish Farming

The most common management practice used by farmers to cope with fish flavor problems is to sample fish flavor periodically and use windows of opportunity to harvest fish between episodes of off-flavor. This practice can be used with some measure of success because the taxonomic composition of pond phytoplankton communities constantly changes, and fish flavor problems occur in sporadic episodes coinciding with the appearance and eventual disappearance of odor-producing cyanobacterial populations. If fish in a pond scheduled for harvest are found to be off-flavor, the farmer can simply wait until the offensive cyanobacterial population disappears naturally, after which time the fish will naturally purge or metabolize the odorous compound. When the odorous compound is completely eliminated from the fish, they can be harvested and sold. The main drawback to this approach is that it is impossible to predict how long the odor-producing cyanobacteria will remain in the pond—they may disappear in a week or they may persist for months.

Many catfish farmers dislike the uncertainties and costs associated with this passive approach and try to manage ponds to reduce the incidence of off-flavor problems. However, off-flavors in catfish have multiple etiologies and problems cannot be eliminated with a single management strategy. As a simple example, off-flavors caused by a small spill of diesel fuel in a pond will not respond to treatment of the pond with algicides. Fortunately, most flavor problems in catfish farming are caused by only two compounds, geosmin and 2-methylisoborneol, both of which are metabolites of certain species of cyanobacteria. Therefore, the overall incidence of off-flavor in pond-raised catfish could be dramatically reduced if odor-producing cyanobacteria were eliminated from ponds. However, even problems related to cyanobacteria—the most thoroughly studied causes of off-flavor in catfish farming—are difficult to control because pond environmental conditions (shallow ponds with high nutrient loading and high turbidity) strongly select for microbial communities dominated by that group of microorganisms.

Aside from the passive "wait-and-see" approach, three general methods have been used to manage off-flavor problems in cultured fish: 1) move off-flavored fish to a "clean" environment and allow sufficient time for fish to "come on-flavor" by purging the odorous chemical; 2) alter the chemical, physical, or biological environment in the pond to discourage development of odorous cyanobacterial communities; and 3) use algicides to prevent or eliminate populations of odorous cyanobacteria. These strategies have been reviewed (*2, 3*) and are discussed in other chapters of this book.

The third alternative described above—using algicides to eliminate noxious cyanobacteria—is analogous to using herbicides to manage weed problems in large-scale terrestrial agriculture. Although the ecological soundness of this approach can be debated endlessly, there is little doubt that chemical control is the approach most favored by catfish farmers, many of whom became familiar with chemical weed control as row-crop farmers before entering catfish aquaculture.

## Using Copper Sulfate to Manage Off-flavors

Copper is required at trace levels for plant growth. Exposure to higher concentrations of copper inhibits growth or kills plants by disrupting a variety of cellular functions, including photosynthesis, respiration, chlorophyll synthesis, and cell division (*4*). In algae exposed to copper, photosynthesis appears to be inhibited to a greater degree than respiration, although overall growth rate is more sensitive than either of the two processes alone (*5*). The most important mechanism of phytotoxicity is binding of copper with sulfhydryl groups of proteins leading to inhibition of critical enzyme systems (*6*).

The algicidal activity of copper salts has been known for centuries, but the first recommendation to use copper sulfate to eliminate algal sources of taste and odor problems in water was made by the German chemist Kronke in 1893 (*7*). Eleven years later, Moore and Kellerman (*8*) made the same recommendation for controlling taste and odor problems caused by algae in water supply reservoirs in

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the United States. At about the same time, Léger (*9*) made an important contribution to the understanding of flavor problems in fish when he speculated that a substance produced by mats of the cyanobacterium *Oscillatoria tenuis* caused muddy flavors in rainbow trout (*Oncorhynchus mykiss*). In 1969, workers in Israel first described the use of copper sulfate to control off-flavors in fish produced in aquaculture (*10*). They reported that the cyanobacterium *Oscillatoria tenuis* caused earthy off-flavors in pond-raised carp (*Cyprinus carpio*) and suggested that the problem could be alleviated by killing the cyanobacteria with copper sulfate or by moving the off-flavored fish to clean water.

Despite a long history of copper sulfate use to control certain flavor problems in both water and fish, the record of its effectiveness in aquaculture, particularly catfish farming, has been inconsistent (*11, 12, 13*). In some instances, copper sulfate treatment is unsuccessful because farmers fail to recognize that not all off-flavors are caused by cyanobacteria or other "algae." This problem was addressed by van der Ploeg et al. (*13*), who proposed a simple decision-making process to help farmers to identify off-flavors that may respond to algicide treatment. First, the presence of 2-methylisoborneol or geosmin is confirmed by taste-testing the fish. Next, a water sample is microscopically examined to verify the presence of cyanobacterial species known to produce one of those odorous compounds. If either piece of evidence is missing, the role of cyanobacteria in the flavor problem is unproven and there is, therefore, no basis for assuming that algicide treatment will help improve flavor quality. If, however, both pieces of evidence are in place, there is reason to believe that fish flavor will improve if the odor-producing cyanobacterial population is killed with an algicide.

Even when there is firm evidence that cyanobacteria are the cause of a particular off-flavor episode, it has proven difficult to formulate a consistently safe and effective treatment protocol for copper sulfate. This difficulty stems from strong interactions between the cupric ion (the principal toxic species of copper to algae; *14, 15*) and certain environmental variables. The most important factors regulating copper toxicity are hardness, pH, and concentrations of dissolved and particulate organic matter. Hardness (specifically calcium) competes with the cupric ion for algal uptake; pH determines the solubility of copper minerals, thereby influencing the concentration of cupric ion in solution; and organic matter can sequester the cupric ion, rendering it temporarily unavailable for uptake by algae. These interactions are largely unquantified, but generally, as the magnitudes of hardness, pH, and dissolved organic matter increase, the toxicity of copper to algae decreases.

The complex nature of copper toxicity means that the use of copper sulfate always carries some risk that environmental factors may make the treatment either ineffective or toxic to nontarget organisms (such as the fish being cultured).

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Potential effects on fish are especially critical because the margin of safety between algicidal and icthyotoxic concentrations is small (less than an order of magnitude).

Copper sulfate treatment rates listed on labels of the commercial algicide range from about 0.06 mg Cu/L (0.25 mg/L as $CuSO_4 \cdot 5H_2O$) to over 0.5 mg Cu/L (2 mg/L as $CuSO_4 \cdot 5H_2O$). Label instructions for the use of copper sulfate as an algicide acknowledge the effect of water chemistry on toxicity but the instructions are usually quite vague regarding those effects. The label may simply warn that toxicity to fish increases in "soft" water and that more chemical may be needed to kill algae in "hard" water.

In a crude attempt to compensate for the effects of water chemistry on copper toxicity, some workers calculate copper sulfate treatment rates (in mg/L as $CuSO_4 \cdot 5H_2O$) by dividing total alkalinity (expressed as mg/L $CaCO_3$) by 100 (*3*). Although this formula acknowledges the effect of pH on copper toxicity (there is a general relationship between total alkalinity and water pH), it does not consider the effects of organic matter and hardness on copper toxicity, and is therefore of limited value as a predictor of safe and effective application rates. Successful use of copper algicides in commercial aquaculture is therefore based largely on trial-and-error.

In the early 1990s, several commercial catfish producers in northwest Mississippi were using copper sulfate to manage algae-related off-flavors with an apparent measure of success. Their practice, which had evolved over many years of experience, involved relatively low doses of copper sulfate applied weekly by suspending a burlap bag filled with copper sulfate in the current produced by an aerator. The following sections describe the results of two studies (*16, 17*) designed to evaluate the effectiveness and consequences of this treatment protocol.

## An Evaluation of Copper Sulfate for Off-flavor Management

Eighteen, 0.4-ha earthen ponds in northwest Mississippi were used in a 3-year study to evaluate the effect of weekly copper sulfate applications on the incidence and economic impact of off-flavors in channel catfish (*16*). The ponds were constructed on alluvial clay soils of the Yazoo-Mississippi River floodplain at Stoneville, MS, and filled with groundwater from the Mississippi River Alluvial Aquifer. Over the study, total alkalinity and total hardness of pond waters varied between 100 and 200 mg/L as $CaCO_3$, with about 70% of the hardness contributed by calcium. Fish production practices were typical of those used in commercial settings. An attempt was made to remove market-sized (> 0.4 kg) fish from all ponds in August or September each year, but actual fish harvest dates depended on acceptability of fish flavor.

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In the first year of the study, nine ponds were randomly selected for treatment; ponds remained in the treatment or control group for all 3 years of the study. Each spring when water temperatures increased above 20°C, the nine ponds in the treatment group were treated weekly with 5.6 kg $CuSO_4 \cdot 5H_2O$/ha (approximately 0.12 mg Cu/L). Treatments were made by placing the required amount of copper sulfate crystals in a double burlap bag that was suspended about 7 m in front of a paddlewheel aerator. The water current produced by the aerator dissolved the chemical and distributed it throughout the pond. The aerator was operated until all the copper sulfate dissolved, which usually required about 2 hours. Treatments were discontinued each fall when water temperatures fell below 20°C. Ponds were treated with copper sulfate 15 times in the first year, 21 times the second year, and 23 times the third year.

Fish flavor quality was assessed at approximately biweekly intervals each summer using sensory analysis methods similar to those used by commercial catfish processors. Flavor quality was described using common descriptors (*18*) and off-flavor intensity was rated on an hedonic scale of 0 to 5, where 0 = no detectable off-flavor and 5 = intense off-flavor. Results of flavor testing in the week before a scheduled fish harvest were used to determine which fish populations would be harvested. In accordance with commercial standards, fish were harvested only from ponds yielding samples with flavor scores of zero. Fish were sampled at approximately biweekly intervals from ponds that were not harvested on the scheduled harvest date. Whenever sampling indicated that fish flavor quality was acceptable, fish in that pond were immediately harvested.

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## Incidence of Off-flavor

Over 3 years, treatment with copper sulfate reduced the point prevalence of ponds with off-flavored fish by about 80%, although treatment success varied among years and on sampling dates within years. Off-flavor was detected in 69 of the 171 samples from control ponds (an overall period prevalence of 40.4%) and 15 of 171 samples (8.7%) from treated ponds.

Most off-flavor sampling occurred before the target harvest dates in late summer of each year. Flavor samples obtained prior to harvest are useful in assessing the effect of copper sulfate treatment on the point prevalence of off-flavor, but in commercial catfish production, off-flavor is important only when present in fish on the desired date of harvest. We chose dates in late summer as target harvest dates because problems with off-flavor are most common at that time of the year (*2*). Off-flavors occurring on the target harvest date caused postponement of harvest from three control ponds the first year, five ponds the second year, and two ponds the third year. In contrast, fish harvest was never delayed by off-flavor in treated ponds over 3 years of the study.

Treatment of ponds with copper sulfate also reduced the duration of off-flavor episodes. The duration of off-flavor events is important because brief postponement of harvest can be tolerated, but long harvest delays have severe financial impacts and increase the probability that fish may be lost to diseases or other causes. The risk of fish loss is especially great when unacceptable flavor quality makes it necessary to hold market-sized fish in inventory through the winter and spring—a time when pond-raised channel catfish are particularly prone to outbreaks of infectious diseases. A total of 24 separate off-flavor episodes were identified by sampling the 18 treated and control ponds over 3 years. Sixteen off-flavor episodes occurred in control ponds and 8 occurred in copper-sulfate treated ponds. Of the 16 off-flavor episodes in control ponds, three lasted less than 4 weeks; seven lasted between 4 and 8 weeks; and six lasted more than 8 weeks. One control pond contained off-flavored fish for 43 weeks. Episodes of off-flavor in ponds treated with copper sulfate were of much shorter duration: of the eight off-flavor episodes in treated ponds, six lasted less than 3 weeks, one lasted 5 weeks, and the longest episode was 6 weeks. Again, none of these episodes occurred when fish were scheduled for harvest.

Not all off-flavors in channel catfish are caused by chemicals synthesized by cyanobacteria, so it is unreasonable to expect that an algicide, such as copper sulfate, will prevent all flavor problems. In this study, only two types of off-flavor were detected by sensory analysis. The most common flavor problem was the characteristic musty flavor caused by 2-methylisoborneol. This off-flavor was the primary cause of 13 of the 16 off-flavor episodes in control ponds and 4 of the 8 off-flavor episodes in the ponds treated with copper sulfate, indicating that copper sulfate treatment was particularly effective in reducing the prevalence of off-flavors caused by 2-methylisoborneol.

The other catfish off-flavor seen in this study was described as "woody." The woody off-flavor is a relatively common flavor problem in northwest Mississippi catfish, although it is much less common than problems due to 2-methylisoborneol (*19*). The chemical cause of the woody off-flavor is not known. Over 3 years, problems with woody flavors were responsible for three episodes of off-flavor in the control ponds and three episodes in treated ponds, showing that copper sulfate had no effect on prevalence of that flavor problem.

## Fish Production

Annual fish harvest averaged over 3 years was 5,349 kg/ha from control ponds and 5,900 kg/ha from ponds treated with copper sulfate. The 9% reduction

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in fish harvest from control ponds was due to three events where infectious diseases caused mass mortalities of fish in populations that should have been harvested earlier were it not for off-flavor. In other words, the inability to harvest fish in a timely fashion from some control ponds exposed fish to a greater risk of loss to diseases. For example, harvest from one control pond was delayed for 43 weeks—from September of one year through April of the following year. In the 2-month period before harvest, a severe outbreak of proliferative gill disease caused large losses of fish from that pond. Only 300 kg of fish/ha were harvested from that pond, which was less than 10% of the average harvest weight from the other control ponds that year. Infrequent, but large, losses of fish also caused greater variation in average annual fish harvest from control ponds (coefficient of variation = 24.3%) than from treated ponds (coefficient of variation = 10.3%).

## Effects on Water Quality

Although copper sulfate treatment had a positive effect on fish flavor quality, it had a negative effect on two key water quality variables. First, average concentrations of nitrite were often higher in copper-treated ponds during the summer treatment period. Mean concentrations of nitrite-nitrogen in treated ponds were above 0.5 mg/L on several dates, and concentrations exceeded 1.5 mg /L in several individual ponds. These concentrations are relatively high, although they are well within the range of values reported for commercial catfish ponds (*20*) and within the water quality tolerance limits for channel catfish, and thus did not affect production.

Ponds treated with copper sulfate also required about 20% more aeration than control ponds during the summer. The negative effects of herbicides on dissolved oxygen concentrations in aquaculture ponds are well known (*3*) but, as indicated below, increased aeration costs were more than offset by increased receipts obtained by higher fish yields from the treated ponds. Other water quality variables showed inconsistent or biologically insignificant relationships to treatment.

## Economics

Enterprise budgets showed that average annual net returns above variable costs were US$1,900/ha for control ponds and $2,720/ha for the treated ponds. Variation in net returns was twice as great for control ponds as for treated ponds, indicating increased stability in production and net returns when off-flavors were managed using copper sulfate. High variation in annual economic performance of control ponds resulted from one or more ponds having very good net returns while

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one or more ponds had extremely poor returns due to protracted episodes of off-flavor. Management tools that reduce variation in economic performance among culture units will improve the predictability of production and input requirements, and thus allow better cash flow projections. Producers, lenders, processors, distributors, and consumers ultimately benefit from a more predictable catfish production system.

## Copper Accumulation in Pond Sediments

Copper is rapidly lost from waters of treated lakes and ponds, eventually becoming incorporated into bottom sediments. Copper does not decompose, as do organic herbicides, so it accumulates indefinitely until the sediments (and their copper burden) are physically transported to another location. Under certain conditions, copper may accumulate in sediments to the point where aquatic organisms are adversely affected. To investigate the possible effects of sediment-bound copper derived from copper sulfate treatments, sediments samples were obtained from the 18 ponds described above after the final fish harvest to determine the accumulation, distribution, and toxicity of copper (*17*)

Sediment samples were collected and processed as described by Han et al. (*17*). Sediment copper was separated into six solid-phase fractions by sequential extraction. This method does not allow precise speciation of solid-phase copper, but it does provide useful information on the gradient of metal binding and potential availability of copper in sediments. Although each extractant in the sequential procedure targets a particular solid-phase component, the fractions are not necessarily well-defined chemically. Nevertheless, the extractant-extractable metal is conveniently referred to by the targeted solid-phase component. In order of increasing binding strength (or decreasing potential bioavailability), the fractions were 1) soluble plus exchangeable copper (EXC), 2) carbonate-bound copper (CARB), 3) copper associated with easily reducible oxides (ERO), 4) organic matter-bound copper (OM), 5) copper associated with amorphous iron oxides (AmoFe), 6) copper associated with crystalline iron oxides (CryFe), and 7) copper in the residual fraction.

The nine treated ponds received 59 applications of 5.6 kg $CuSO_4 \cdot 5H_2O$/ha over 3 years, totaling 85.2 kg Cu/ha. Copper sulfate was not applied to the nine control ponds in that period and there was no history of copper sulfate use in those ponds in the 15 years since they were constructed. Accordingly, the mean (± SD) total extractable copper concentration in treated pond sediments (172.5 ± 35.2 mg/kg) was much greater than in control pond sediments (36.1 ± 11.7 mg/kg). The copper concentration in sediments from control ponds is similar to that in

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native terrestrial soils at sites near the ponds (mean 33.7 mg/kg; range 29-39 mg/kg)(*21*).

Sequential extraction showed large differences in the nature and potential availability of solid-phase copper in sediments from treated and control ponds (Table I). Copper concentrations in fractions with the greatest potential bioavailability were much greater in treated pond sediments (sum of mean water-soluble Cu and Cu in EXC fraction = 6.52 mg/kg) than in control pond sediments (water-soluble Cu plus Cu in EXC fraction = 0.69 mg/kg). Sediment copper in treated ponds was mainly present in the CARB (31.6%) and OM (31.1%) fractions. The large proportion of copper in the CARB fraction is reasonable because the water used to fill the ponds sampled in this study had moderately high alkalinity (100 to 200 mg/L as $CaCO_3$) and an alkaline pH (7.8-9.5) that would promote initial precipitation of copper as a carbonate. Following initial precipitation, copper in treated pond sediments may redistribute over time from the CARB fraction into the AmoFe fraction (which represented 22.1% of the total). The large proportion of copper in the OM fraction can be explained by uptake of copper by phytoplankton, followed by sedimentation of dead phytoplankton and incorporation into the pond bottom soil.

**Table I. Distribution of copper among solid-phase fractions (as percentages of total extractable copper) in sediments treated and not treated (control) with 59 additions of 5.6 kg $CuSO_4 \cdot 5H_2O$/ha over 3 years (17)**

| *Copper Fraction* | *Treated Sediment* | *Untreated Sediment* |
|---|---|---|
| EXC | 3.4 | 1.2 |
| CARB | 31.6 | 15.8 |
| ERO | 0.4 | 0 |
| OM | 31.1 | 21.4 |
| AmoFe | 22.1 | 28.3 |
| CryFe | 7.0 | 20.2 |
| RES | 4.3 | 13.0 |
| Total (mg/kg by sum) | 165.6 | 32.2 |
| Total (mg/kg by analysis) | 172.5 | 36.1 |

In contrast, most of the sediment copper in control ponds was firmly bound in stable fractions—over 80% of the total copper was in the AmoFe, OM, CryFe, and RES fractions (Table I). Overall, these results suggest that copper recently incorporated into sediments as a result of treatment with copper sulfate will ultimately redistribute to fractions of low bioavailability.

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Relative binding intensity ($I_R$) is an index of the proportion of metal found in increasingly stable fractions during sequential extraction procedures (*22*). Values of $I_R$ for the procedure used here can range from 0.002 to 1. Low values indicate that most of the metal is present in labile, easily extractable fractions. High values (near $I_R = 1$) indicate that most of the copper is present in stable fractions resistant to extraction. The value of $I_R$ for copper in control pond sediments (0.51) was 1.5 times that for treated pond sediments (0.34), which is consistent with the general observation that copper added to soils redistributes over time from labile fractions to more stable, firmly bound fractions (*23*).

Differences in the distribution of copper among sequentially extracted solid-phase fractions and subsequent calculation of relative binding intensity indicate greater potential bioavailability of copper in sediments from treated ponds. Nevertheless, when amphipods (*Hyallela azteca*) and cattail (*Typha latifolia*) seedlings were exposed to rewetted sediments from treated and control ponds, there was no indication of toxicity (*17*).

## General Comments

The copper sulfate treatment rate evaluated above (biweekly treatments with 5.6 kg $CuSO_4 \cdot 5H_2O$/ha) was developed through trial-and-error by catfish farmers in northwest Mississippi. Toxicity of copper to phytoplankton and fish is strongly affected by water quality, and safe, effective treatment rates may differ for ponds in other regions.

Although copper sulfate can be a useful tool to manage fish flavor problems, its use will adversely affect some water quality variables. Most important, ponds treated with copper sulfate will have higher nitrite concentrations and require more supplemental aeration than untreated ponds.

Copper concentrations in water decline rapidly after addition of copper sulfate (*24, 25*) because copper is taken up by phytoplankton or precipitated from pond water as copper oxides. Nearly all the copper added after treatment will eventually become incorporated into sediments. The work reported in this paper and elsewhere (*23, 25*) shows that copper initially accumulates in sediment or soil fractions that are potentially bioavailable. However, the amount of copper that accumulated in catfish pond sediments after addition of 85.2 kg Cu/ha over 3 years did not result in sediment toxicity as assessed in bioassays using amphipods and cattail seedlings. This result should be interpreted with care because catfish ponds may be treated for longer periods, resulting in greater sediment copper burden. Also, bioassay conditions (rewetted, dry sediments were used) and the test organisms selected for the bioassay may mask subtle effects of sediment-bound copper, such as those on sediment microbial activity.

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Because copper accumulates in pond sediments, very little metal will be transported to waters receiving pond effluents. This was verified in yet another study (*26*) using the nine copper-treated ponds described in this chapter. In that study, sediments were sampled upstream and downstream from the outfall of ponds treated with copper sulfate for 3 years. Sediment copper concentrations did not differ at the two sites, demonstrating that little or no copper was being transported out of the treated ponds. Furthermore laboratory tests did not demonstrate sediment toxicity related to copper, suggesting that use of copper sulfate to manage catfish off-flavors should pose little risk to the environment outside the pond.

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## Chapter 11

# Microbial Algicides: Potential for Management of Cyanobacteria That Cause Off-Flavor in Aquaculture

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The economic importance of off-flavor in aquaculture and evidence that species of *Anabaena* and *Oscillatoria* are responsible for most of this off-flavor indicate the need to develop better management practices for cyanobacteria. Microbial agents that are pathogenic or antagonistic to cyanobacteria are widely distributed and significant opportunities exist to develop these agents as microbial algicides to help manage cyanobacteria in aquaculture. Microbial algicides can be effective, highly selective, and safe to the environment and nontarget species. Results of preliminary evaluations indicate that a bacterium isolated from a commercial catfish pond in Louisiana could be a potential microbial algicide for species of *Anabaena* and *Oscillatoria*.

Off-flavor is one of the most serious problems in aquaculture, particularly in the catfish industry (*1-4*), where annual losses attributed to off-flavor have been estimated at $12 million (*5*) to $50 million (*6*). Recent reports review the various causes of off-flavor and the impact of off-flavor in aquaculture, particularly in the commercial production of channel catfish (*Ictalurus punctatus*) (*4, 6-8*).

Compounds of microbial origin are responsible for most of the off-

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

flavor associated with economic losses in the catfish industry (*4*). Several species of actinomycetes, algae, and cyanobacteria are known to produce a number of off-flavor compounds (*9-17*). In the catfish industry, the two most important off-flavor compounds of microbial origin are geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneol (MIB) that are produced by cyanobacteria. Geosmin off-flavor has been associated with species of *Anabaena*, while MIB off-flavor has been linked to *Oscillatoria perornata*, a planktonic species that was previously reported as *Oscillatoria* cf. *chalybea* (*4, 7, 8, 18-24*). Because cyanobacteria are the primary sources of off-flavor compounds that are produced during commercial production of channel catfish (*1-4, 6-8, 16-25*), management of cyanobacteria has been proposed as a means to control off-flavor (*4, 7, 23, 25*). Management practices for control of cyanobacteria have been reviewed recently (*4*), and these include: nutrient manipulation, the use of filter feeding fish, applications of naturally-occurring compounds, and the use of algicides. Copper compounds, the only algicides currently registered for aquaculture in the United States, are nonselective and are toxic to a wide range of algae and cyanobacteria (*4, 7*). At present, there are no methods for selective control of cyanobacteria, particularly for *Anabaena* spp. and *O. perornata.*

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## Microbial agents that lyse cyanobacteria

A number of microbial agents such as viruses (cyanophages) (*26-50*), bacteria (*51-59*), actinomycetes (*47, 60*), and fungi (*61-65*) have been reported to lyse cells of cyanobacteria. These reports indicate that lytic agents of cyanobacteria are widely distributed in soil and aquatic environments. The concept of using cyanophages to control cyanobacteria was proposed by Safferman and Morris in 1964 (*43*), and algal viruses were discussed as having desirable characteristics for use as algicides (*28*). Research concerning the potential use of cyanophages and other microbial agents to control cyanobacteria has been reviewed several times (*28, 31, 38, 39, 47, 52, 54, 65*). These reports indicate the complex nature of the interactions between cyanobacteria and lytic microbial agents. The roles of the various lytic agents in the population dynamics of cyanobacteria are not well defined (*52, 53, 55*). However, evidence indicates that lytic agents can influence shifts in phytoplankton communities (*28, 30, 34, 36, 37, 39, 41, 44, 50*). The modes of action of the various types of lytic agents have been reviewed (*31, 47, 64*). The potential manipulation of some microbial agents to control cyanobacteria is viewed as a valid concept (*31, 39, 41, 47, 65*).

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Development of microbial algicides to control cyanobacteria in aquaculture has economic and scientific merit. A number of microbial agents have been reported to lyse species of *Anabaena* (*28, 30, 32, 33, 37, 39, 41, 53, 54, 57, 59, 64, 65*) and *Oscillatoria* (*49, 53, 59, 65*). Because lytic agents of cyanobacteria are widely distributed, there are opportunities to isolate additional microbial agents that lyse cyanobacteria, particularly species of *Anabaena* and *Oscillatoria*, that cause off-flavor. The relatively narrow host ranges that are exhibited by many of these lytic agents would minimize risks to nontarget species. While not all lytic agents are pathogenic, the high population densities that are often associated with species of *Anabaena* and *Oscillatoria* would be conducive to the rapid reproduction (replication) and spread of lytic agents that are pathogenic to these cyanobacteria. Because of the economic losses associated with off-flavor in aquaculture, there would appear to be significant potential economic benefits associated with development of microbial algicides to control *Anabaena* spp. and *O. perornata.*

Pilot scale evaluations of experimental microbial algicides should include both technical and economic feasibility studies. Technical feasibility evaluations include:

- Demonstration that the microbial algicide will control the target species under pond conditions;
- Demonstration that the microbial algicide is safe to crop and nontarget species;
- Determination of the effective dosage rates of the experimental microbial algicide;
- Determination of the effects of treatments on dissolved oxygen;
- Determination of the influence of pH, water temperature, and water chemistry on the effectiveness of the experimental microbial algicide.

Economic feasibility evaluations assess control of the target species using amounts of inoculum that can be mass-produced, formulated, and applied in a manner that would be acceptable and affordable to the end user.

## Examples of research to develop microbial algicides

As a result of the author's ongoing research to isolate and evaluate microbial agents to control cyanobacteria associated with off-flavor in commercial production of channel catfish, several lytic agents have been isolated (*69*). The isolation process is a modification of a method to produce and formulate microbial herbicides (*70*), and it can be easily adapted to isolate lytic agents for specific algae or cyanobacteria.

## Isolation of Lytic Agents for Cyanobacteria

Unialgal suspension cultures of the target species were incubated in flasks on an orbital shaker (*59*). Uniform suspension cultures, including cultures of filamentous mat-forming species, were established after a series of serial transfers. Each cyanobacterium growing in suspension culture was immobilized in sodium alginate gel beads (*69, 70*).

In laboratory studies, the immobilized cyanobacteria were very efficient for detection of cyanophages in aqueous preparations (*63*). The virus concentration increased in the gel beads in proportion to the concentration of the immobilized host cells, providing a basis for production, concentration, and storage of cyanophages (*63*).

To isolate lytic agents from pond water, the immobilized cyanobacteria were placed in a sampling device constructed of clear plastic or polyvinyl chloride (PVC) pipe, transported to a collection site, and floated on the surface of the water or suspended in the water column for 2 to 5 days. The immobilized cyanobacteria were then placed onto lawns or into suspension cultures of the respective target organisms. The presence of lytic agents was indicated either by the development of zones of clearing in the lawns or by the destruction of cyanobacteria in the suspension cultures.

This process allows continuous sampling (2 to 5 days) of water for the presence of lytic agents. As pathogenic (lytic) agents replicate in the high density of immobilized host cells, many of these agents are retained inside the gel beads, thus increasing the chances for detection (*69*). This process has been used to isolate viruses, bacteria, and fungi that lyse cells of several algal and cyanobacterial species, including species of *Anabaena* and *Oscillatoria* (*59, 63*).

Daft et al. (*30*) described a similar method that used pieces of wood to float filter paper that had the target cyanobacteria attached. Other methods to isolate lytic agents of cyanobacteria have been widely used with a high degree of success (*26, 30, 31, 33, 35-37, 40, 44, 45, 53, 54, 57, 65*).

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## Laboratory Experiments to Evaluate Bacterium SG-3

Bacterium SG-3 is a lytic agent that was isolated from immobilized cells of *O. perornata* that had been suspended in a commercial catfish pond for 4 days. The host range of bacterium SG-3 includes a number of *Anabaena* and *Oscillatoria* species, including *O. perornata* (*59*). The bacterium produced 3 x $10^9$ plaque forming units (PFU)/mL when cultured in BG-11 medium containing 6 g/liter tryptic soy broth and assayed in lawns of *Anabaena* sp. (ATCC 27898). The number of PFU was determined using a procedure modified for assay of cyanophages (*42*). Bacterium SG-3 has been deposited as NRRL B-0043 in the

ARS Patent Culture Collection, Peoria, IL, and patented as a microbial algicide (*68*).

## Field Experiments to Evaluate Bacterium SG-3

Water from commercial catfish ponds with blooms of *Oscillatoria* spp. was transported using a 4000-L tank/trailer (Figure 1) and 568-L (150 gal.) were dispensed into each of nine 757-L (200 gal.) polypropylene tanks. Each tank was supplied with aeration and stocked with 10 channel catfish fingerlings. This process resulted in nine experimental units that had uniform populations of phytoplankton. Treatments were randomized among the experimental units, and each treatment was replicated three times. Uniform populations of the *Oscillatoria* spp. were difficult to maintain in the tanks for more than 5 or 6 days. Consequently, bacterium SG-3 was tested at relatively high concentrations so that the treatments could be evaluated within this period of time. Inoculum for bacterium SG-3 was grown in the laboratory and applied to the pond water in the tanks. Filament counts of *Oscillatoria* species were determined using a Sedgwick-Rafter counting chamber.

In the first experiment, 5.7-L of bacterium SG-3 inoculum ($2 \times 10^9$ PFU/mL) were applied to 568-L of pond water in each of three tanks. Uninoculated growth medium (5.7-L of BG-11 containing 6 g/liter tryptic soy broth) was applied to pond water in three tanks. Controls consisted of untreated pond water in three tanks. The initial concentration of *O. perornata* was approximately 3,900 filaments/mL of pond water. Within 2 days after inoculation, no filaments of *O. perornata* were observed in the water that was treated with the bacterium (Figures 2 and 3). A *Microcystis* sp. became the dominant phytoplankton species in the pond water treated with bacterium SG-3 (Figure 2). These results are consistent with laboratory studies that indicated *Microcystis aeruginosa* (UTEX 2063) was resistant to the bacterium (59).

In a second experiment, the dominant phytoplankton species was an *Oscillatoria* sp. (tentatively identified as *O. agardhii*), with an initial concentration of approximately 2,800 filaments/mL of pond water (Figure 4). Bacterium SG-3 inoculum ($2 \times 10^9$ PFU/mL) was applied at rates of 5.7-L (1:100) or 1.9-L (1:300) per 568-L of pond water. Controls consisted of untreated pond water in three tanks. Within 1 day after treatment, the numbers of filaments of the *Oscillatoria* sp. were reduced <10% and 87%, for inoculum rates of 1.9-L and 5.7-L, respectively. Within 2 days after treatment, no *Oscillatoria* sp. filaments were observed in pond water treated with either amount of bacterium SG-3 inoculum. In contrast, there were approximately 3,700 *Oscillatoria* sp. filaments/mL of untreated pond water (Figure 4).

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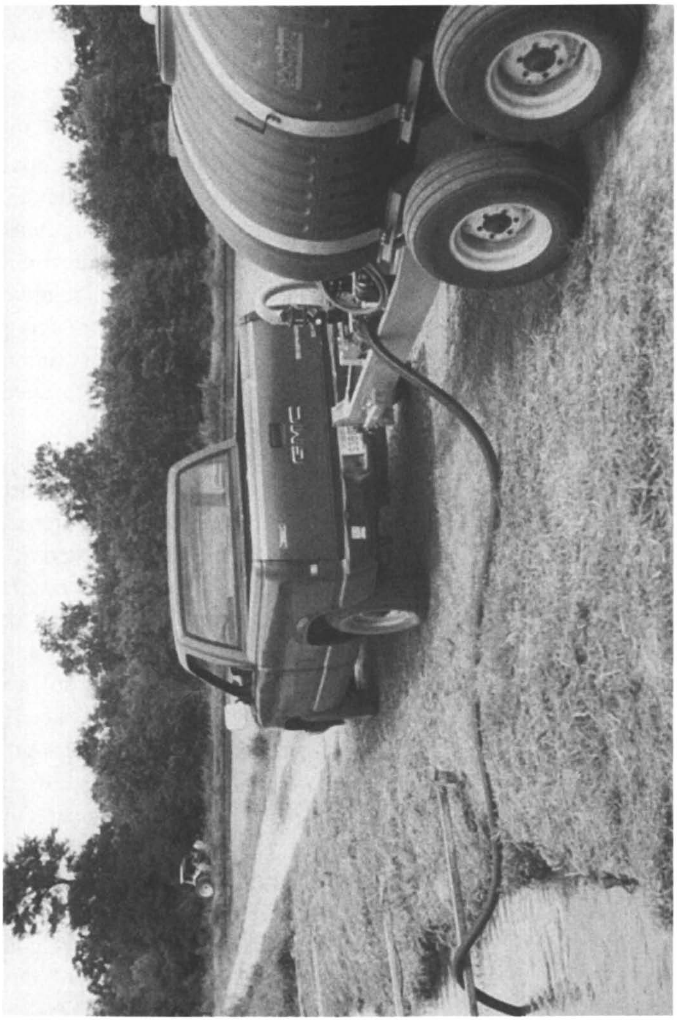

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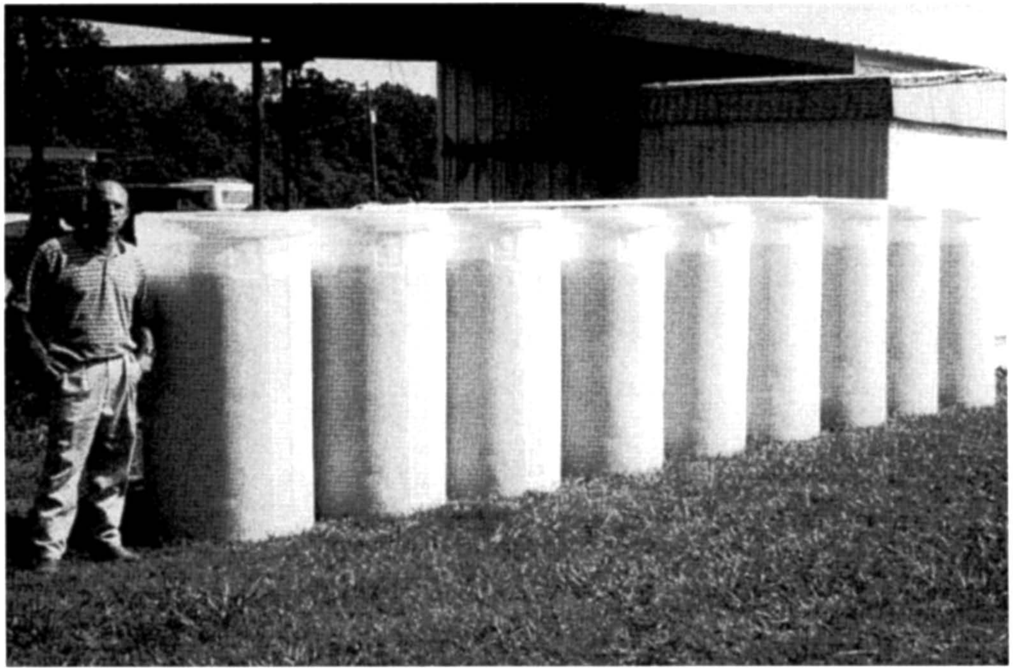

Figure 1. Water from a commercial catfish pond was transported in a 4,000-L tank/trailer (top), and 568-L were dispensed into each of nine 757-L polypropylene tanks (bottom).

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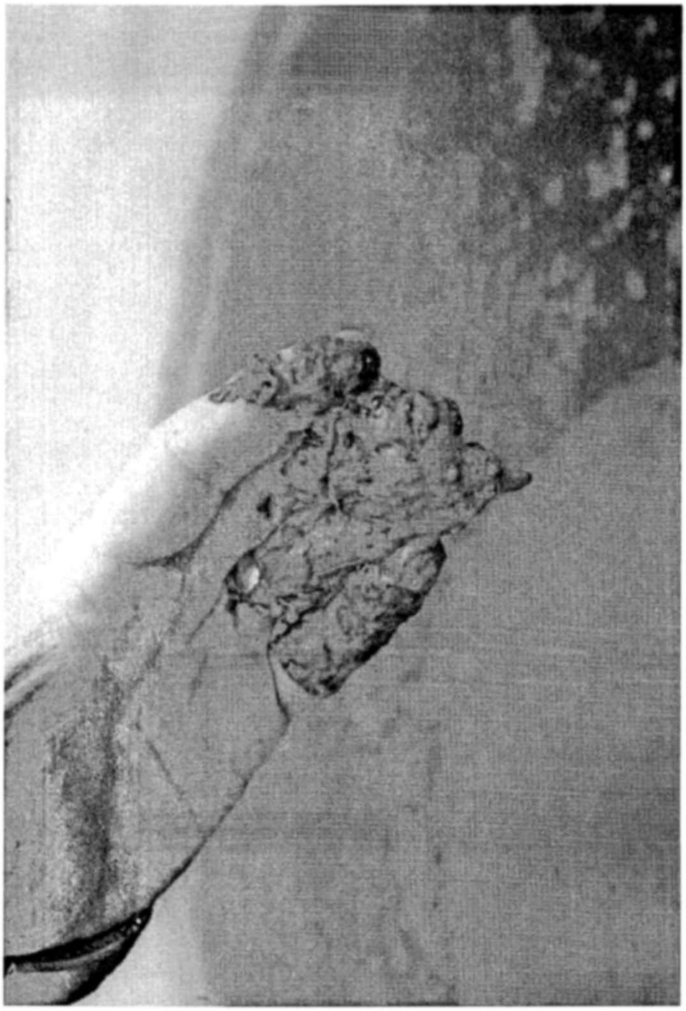

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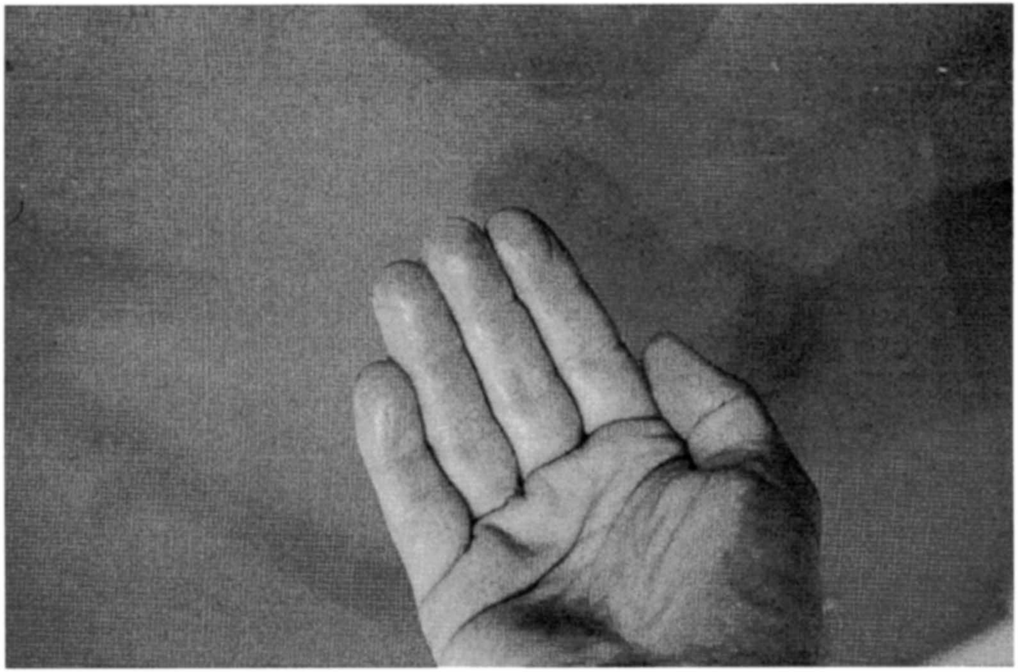

Figure 2. Water (568-L) from a commercial catfish pond that exhibited a bloom of *Oscillatoria perornata* was treated with 5.7-L of bacterium SG-3 inoculum (2 x $10^9$ PFU/mL). The photographs were made 5 days after treatment. When compared to the untreated control (top, left), note absence of floating clumps of cyanobacteria in water treated with bacterium SG-3 (top, right). On the bottom are photomicrographs of water from an untreated control (left) and water treated with bacterium SG-3 (right). Note absence of *0. perornata* filaments in the treated water. A *Microcystis* sp. became dominant in the treated water.

*Continued on next page.*

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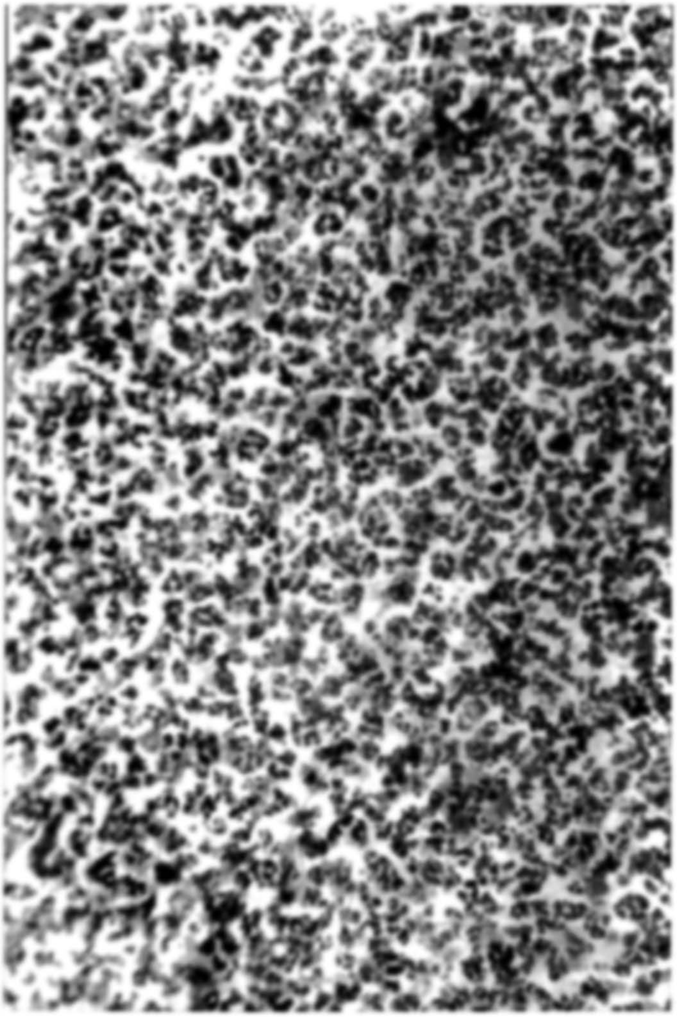

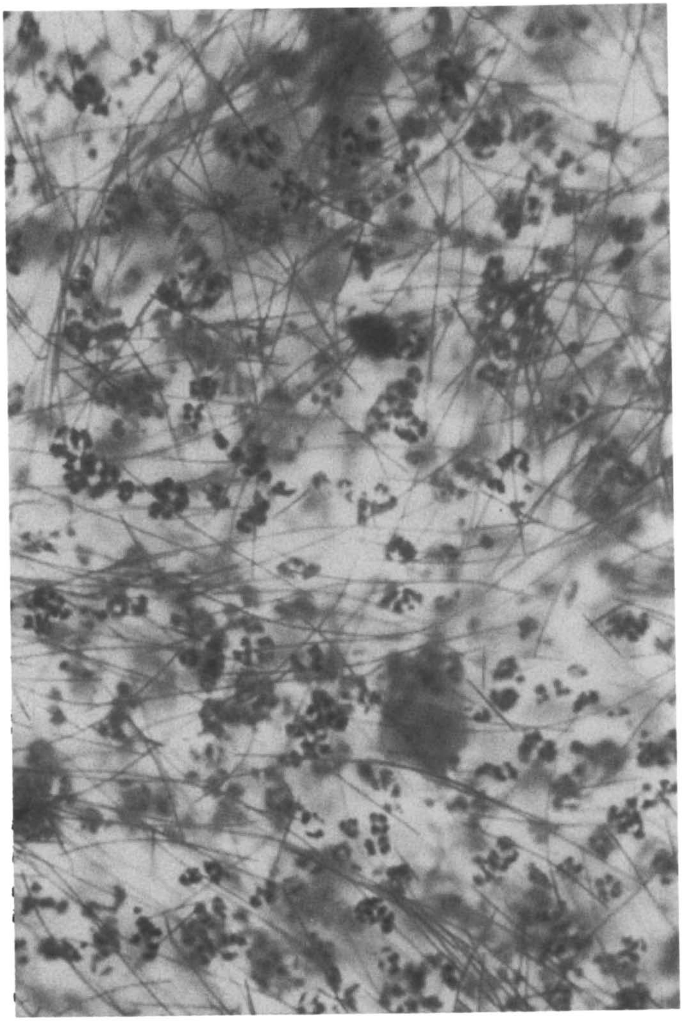

Figure 2. *Continued.*

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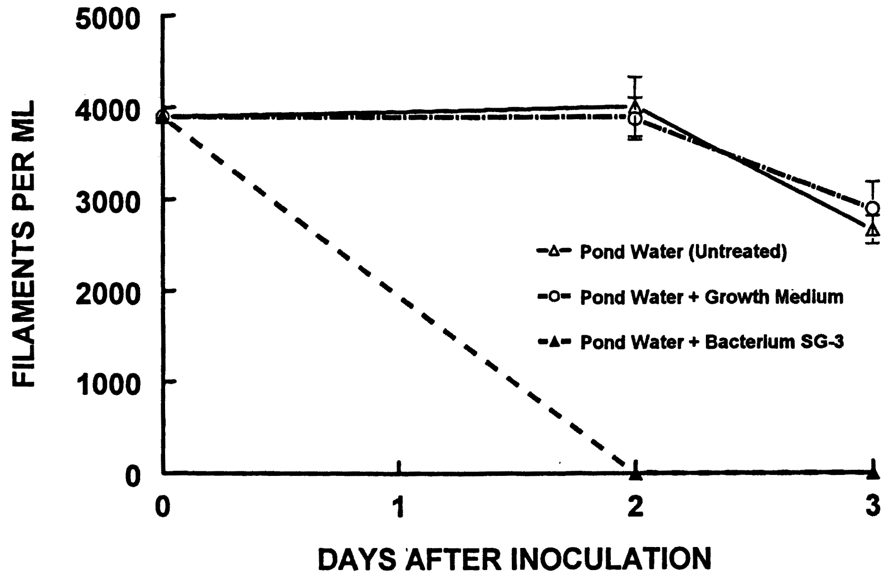

Figure 3. Response of *Oscillatoria perornata* to bacterium SG-3 in tank studies. Water (568-L) from a commercial catfish pond was placed in each of nine polypropylene tanks and treated with either 5.7-L of bacterium SG-3 inoculum (2 x $10^9$ PFU/mL) or 5.7-L of uninoculated growth medium (BG-11 medium containing 6 g/liter tryptic soy broth). Each value is the mean ± SEM from three replicates.

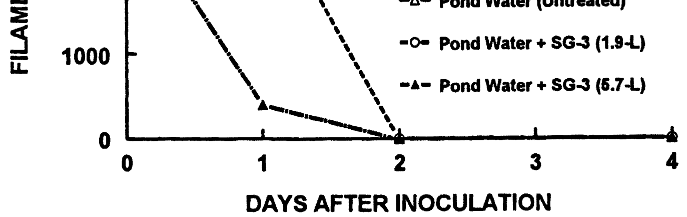

Figure 4. Response of an *Oscillatoria* sp. (tentatively identified as *0. agardhii)* to bacterium SG-3 in tank studies. Water (568-L) from a commercial catfish pond was placed in each of nine polypropylene tanks and treated with either 5.7-L or 1.9-L of bacterium SG-3 inoculum (2 x $10^9$ PFU/mL). Each value is the mean ± SEM from three replicates.

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In the second experiment, the numbers of PFU were assayed in lawns of *Anabaena* sp. (ATCC 27898) following application of inoculum to pond water (Figure 5). The observed increase in the number of PFU within 1 day after application of inoculum indicates that bacterium SG-3 reproduced in water from a commercial catfish pond that contained large numbers of other microorganisms. [Bacterial cell counts for marine and freshwater systems have been estimated to be 1 x $10^6$ bacterial cells/mL of water (*52*).] The decline in the number of PFU/mL of pond water within 4 days after application of inoculum could be associated with the decline in the number of filaments of the *Oscillatoria* sp., as has been observed in laboratory studies (59).

In both of these field studies, the high levels of inoculum of bacterium SG-3 resulted in rapid lysis of the *Oscillatoria* spp. However, the aeration that was supplied to the tanks maintained the dissolved oxygen levels near saturation. Laboratory studies indicate that the rate of lysis of *Anabaena* and *Oscillatoria* species can be controlled by varying the inoculum levels of bacterium SG-3 (*59*). If these results can be verified in pond experiments, then lower inoculum levels could result in slower rates of lysis for the host cyanobacteria, thus minimizing the potential for oxygen depletion.

There was no evidence that bacterium SG-3 harmed the catfish fingerlings. Some mortality (<10%) was occasionally observed, but the dead fish were randomly distributed among control and treatment tanks. In laboratory studies, channel catfish fingerlings exhibited no apparent adverse effects when they were maintained in water containing high concentrations of bacterium SG-3 (H. L. Walker, unpublished).

## Involvement of commercial interests

Development and implementation of microbial algicides into aquaculture production systems will require collaboration between the public and private sectors. Microbial agents that are developed and marketed for control of cyanobacteria or algae will be classified by the United States Environmental Protection Agency (USEPA) as microbial pesticides. Consequently, these microbial algicides are subject to the guidelines for evaluation and registration that are specified by the Federal Insecticide Fungicide Rodenticide Act (FIFRA), as amended in 1988. These guidelines specify that pilot-scale evaluations for each experimental microbial algicide must be limited to one surface acre of water per year, unless an Experimental Use Permit (EUP) or special approval is obtained from the USEPA. In addition, tolerance levels must be established for aquaculture food crops that are exposed to experimental microbial algicides. Registration requirements, as specified in

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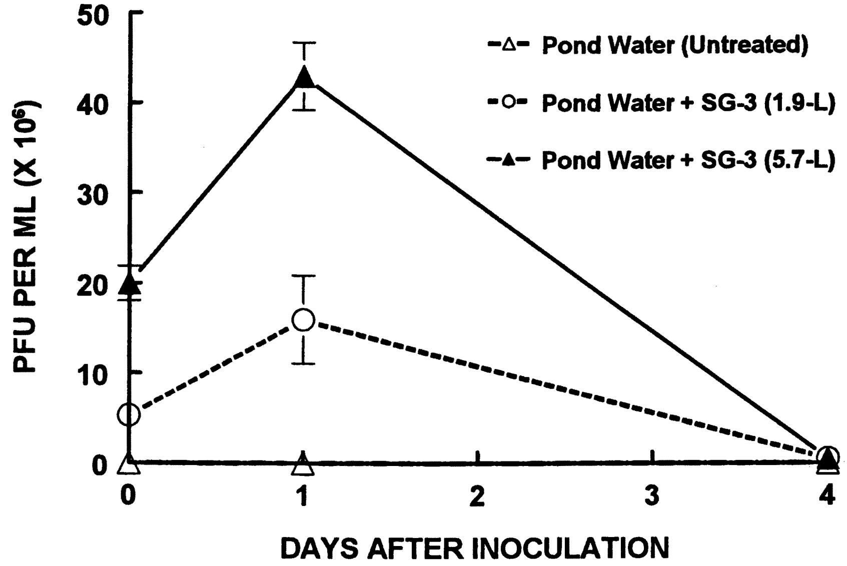

Figure 5. Numbers of plaque forming units (PFU) produced by bacterium SG-3. The numbers of PFU/mL of pond water were determined from assays in lawns of *Anabaena* sp. (ATCC 27898). Water (568-L) from a commercial catfish pond exhibiting a bloom of *Oscillatoria* sp. (tentatively identified as *0. agardhii)* was placed in each of nine polypropylene tanks and treated with either 5.7-L or 1.9-L of bacterium SG-3 inoculum (2 x $10^9$ PFU/mL). Each value is the mean ± SEM from three replicates.

FIFRA, will emphasize safety to humans and other nontarget species, and persistence in the environment. A review of FIFRA and other laws governing biological control agents has been recently published (*67*).

Decisions to pursue commercial development will be influenced by the potential for implementation of the microbial algicide into aquaculture production practices, the potential benefits to the end user, and the projected costs associated with registration, product development, and marketing.

Patent protection could greatly influence decisions to pursue commercial development, because such protection would allow time for a company to develop a product and recover investment costs. Decisions to patent are usually made early in the evaluation process, and these decisions are coordinated with public disclosure of the invention. Patent applications must be filed in the United States within one year after public disclosure of an invention; however, in some other countries patent applications must be filed before public disclosure of the invention. Collaboration between the public and private sectors is enhanced as patents from the public sector are licensed for commercial development.

## Conclusions

The management of phytoplankton in aquaculture ponds has been compared to the control of terrestrial weeds (*4*). Likewise, the development of microbial algicides for aquaculture can be compared to the development of microbial herbicides for terrestrial weeds. One of the major problems related to the development of microbial herbicides for terrestrial weeds has been the restricted host ranges of the biological control agents (*66*). The costs of patent protection, registration, product development, production, and marketing can be prohibitive for a microbial herbicide that will control only one or two species of weeds.

Many of the limitations related to the control of terrestrial weeds would not be applicable for microbial algicides used in aquaculture ponds. If microbial algicides could be applied behind aerators, with subsequent uniform distribution in the ponds, application costs would be minimal when compared to similar costs for row crops. Compared to application of microbial herbicides to row crops, the improved logistics and economic factors would permit application of larger quantities of inoculum to aquaculture ponds. Because of the economic impact of off-flavor, there is evidence that microbial algicides that control only selected species of cyanobacteria, particularly *Anabaena* spp. and *Oscillatoria* spp., could be viable commercial products. Cyanobacteria in general are viewed as a problem group in aquaculture (*4*), and microbial algicides with host ranges

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that include additional species of cyanobacteria could have enhanced market potential. Mixtures of lytic agents could further increase the spectrum of algicidal activity (*31, 47*).

However, a number of challenges must be addressed for successful integration of microbial algicides into management practices in aquaculture. Many of the factors that influence algicidal activity of microbial agents have been reviewed (*31, 38, 39, 47, 53*). Factors that could pose challenges for the development of microbial algicides include the following: 1) the temperate/lytic characteristics of some cyanophages (*28, 38, 39, 72*); 2) development of resistant strains of cyanobacteria (*27, 31, 39, 47*); 3) microbial agents for which algicidal activity is dependent on substances that would be diluted to ineffective concentrations by pond water (*31, 47*); 4) pH ranges that could inactiviate or inhibit certain types of lytic agents (*53*); 5) requirements for monovalent or divalet cations that could prove to be limiting (*31, 38, 39*); 6) temperature or light interactions that could influence algicidal activity (*38, 39, 53, 71, 73*); 7) requirements for excessive amounts of inoculum (*47*); 8) problems related to large-scale inoculum production, particularly for the obligate parasites (*31, 47*); and 9) the possibility that bacteriophages in pond water could inhibit the algicidal properties of lytic bacteria, such as bacterium SG-3.

Even if a microbial algicide is effective for the control of problem species of cyanobacteria, there will not be an immediate reduction in off-flavor of the fish. Most of the geosmin and MIB is located inside living cells of cyanobacteria, and lysis of these cells results in temporary increases in off-flavor compounds in the water (*4*). However, these off-flavor compounds dissipate in the absence of the organisms that produce them (*4*).

In spite of these challenges, there are opportunities to develop microbial algicides that could provide the aquaculture industry with additional tools to manage cyanobacteria and off-flavor. Just as integrated pest management practices are important for weed control in terrestrial crops, control of off-flavor in aquaculture will require integration of different management practices (4). In the future, these management practices could include the use of microbial algicides.

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# Chapter 12

# Biological Control of Off-Flavor Cyanobacteria

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Biological controls tend to be highly selective, environmentally-friendly and may produce a marketable product. Although some protozoa specialize in feeding on cyanobacteria, filter-feeding macroorganisms (FFM) more easily remove the 10% biomass required daily and may have economic potential. Ten FFM species and their hybrids were evaluated for removal of naturally-occurring off-flavor cyanobacteria in 500-L mesocosms at the Aquaculture Research Station of the University of Arkansas at Pine Bluff (UAPB). Nile and blue tilapias and silver carp were effective in removing 90% of the two major groups of problem cyanobacteria (*Anabaena* spp.and O. cf. *chalybea*) in 48 h. Threadfin shad were also effective in removing *Anabaena* and need further evaluation for judging effects on *O.* cf. *chalybea.* Experimental, 0.045-ha pond evaluations, market potential and the availability of genetically male strains (GMT) supported Nile tilapia as the species of choice in biological control. Nile tilapia in 1-ha blue catfish fingerling ponds eliminated (or reduced below problem levels) cyanobacteria of concern in 30 d following stocking of 5,000 fingerling tilapia/ha. The tilapia net cage system in ponds of free-swimming, fed channel catfish has the potential to control off-flavor, while adding up to 2,000 kg/ha of valuable fish production.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

## Introduction

Off-flavors in aquaculture are predominately caused by cyanobacteria, also known as blue-green algae. The cause of musty flavors is 2-methylisoborneol (MIB) which is a metabolic by-product of the large, filamentous cyanobacterium *Oscillatoria* cf. *chalybea/Planktothrix perornata*. Actinomycetes also produce MIB. The other common off-flavor is described as earthy, and caused by geosmin. Geosmin is a metabolic by-product commonly produced by filamentous cyanobacteria of the genus *Anabaena*, including several straight chain forms and the coiled *A. circinalis*. The off-flavors woody and pine (from high MIB) are assumed to be related to cyanobacteria. We have found pigpen off-flavor associated with sulphur bacteria and cyanobacteria scums. Off-flavor occurrence has been correlated with cyanobacteria dominance (*1*) and high feeding levels and water temperatures (*2*). Removing problem species of cyanobacteria is the goal of preharvest off-flavor management. Cropping of 10% of the phytoplankton daily by physical (flushing), chemical or biological means has been suggested as a management approach (*3*). Water replacement by flushing is not feasible in large impoundments and with discharge regulations on pollutants. Control by approved chemical herbicides has been investigated and developed using copper sulfate (*4*).

Biological controls by natural consumers of cyanobacteria are thought to hold promise (*5*) and have been investigated in a multi-institution grant of which this investigator considered the use of filter-feeding macroorganisms (FFM) such as fishes and clams. Microorganism consumers of *Oscillatoria* spp. include nassophorine ciliates (protozoa) of the genus *Nassula* (*6*). However, sufficient densities to crop 10% of phytoplankton daily would appear to be difficult to achieve at sustained levels.

Worldwide, 17 species of fishes are known to include substantial percentages of phytoplankton in their diet (*7*) and, thus, are candidates to control off-flavor causing algae. To be effective in this regard, they must filter and digest the cyanobacteria genera *Oscillatoria* and *Anabaena*. The tilapia genus *Oreochromis* includes several species adapted to feeding on cyanobacteria in the naturally eutrophic lakes of East Africa (*8*). Unlike most filter-feeding fish, these fishes do not use gill rakers and rather entangle phytoplankton with mucus from the gills, and a stomach pH below 2 allows digestion of cells such as *Microcystis* (9).

Several filter-feeding fishes have been found to experimentally control one or both of the major off-flavor cyanobacteria genera. Nile tilapia (*O. niloticus*) in aquaria filtered 92-9 % of *O.* cf. *chalybea* and *A. sphaerica* in 24 h (*10*). Stocking was 1.2 g/L for small fish (8 g) and 2 g/L for medium fish (30 g). Blue tilapia eliminated larger algae, including cyanobacteria, in unfertilized concrete pools (*11*), fertilized ponds and pools, fed catfish experimental ponds and in laboratory filtration trials (*12*). Blue tilapia (*O. aureus*) in polyculture with channel catfish *Ictalurus punctatus* reduced the occurrence of off-flavors in one study (*13*), but had no effect on off-flavor while producing rotting off-flavors (when the tilapia died

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due to lethal low temperatures) in another polyculture study with channel catfish (*5*). Tilapia were not confined in these studies and had access to feed.

Blue tilapia and common carp *Cyprinus carpio* have been reported to reduce off-flavors in Israeli reservoirs (*14*). Anecdotal reports of control by threadfin shad *Dorosoma petenense* have been made by fish farmers. I have examined these candidates and others in mesocosm, and in experimental and production pond evaluations using a system of free swimming primary species and a sequestered (cage/net pen) filter-feeder.

## Filter-Feeding Macroorganism Evaluations

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### Mesocosm Studies

Mesocosm studies using 500-L outdoor pools were conducted to determine filtering abilities of candidate FFM species for off-flavor algae control. The design was based on the aquaria filtration studies of Perschbacher and Lorio (*10*). In this study, Nile tilapia were presented with pond plankton containing cyanobacteria of concern. Three aquaria served as controls and three contained tilapia. Aquaria were stirred prior to sampling. A 100-ml sample was sedimented for one week following concentration and evaluation methods of APHA (*15*). The supernatant was removed and the concentrated sample measured. One ml was examined under 150X in a Sedgwick-Rafter counting cell. Ten fields were counted with cyanobacteria identified to species using Cocke (*16*). Although *O.* cf. *chalybea* (U.S.) and *O. raciborskii* (Japan) have been suggested to be *Planktothrix perornata* var. *attenuata* used in Australia (5), *O.* cf. *chalybea* as the earliest designation for this alga is retained in this paper. The number of filaments/colonies were determined per ml and % change and t-test comparisons (0.05 level) made aganist controls. Chlorophyll *a* indicated changes in biomass (also of interest in algal control). Pheophytin *a* was measured to provide both an indication of physiological status of the algal populations and of algal grazing (*15*).

#### *Bighead Carp and Gizzard Shad*

Bighead carp *Hypophthalmichthys nobilis* were evaluated in mesocosm with water from experimental production ponds on the UAPB Aquaculture Research Station. The off-flavor algae presented were *O.* cf. *chalybea, Anabaena sphaerica* and A. *circinalis*. After 4 days, no significant reductions were observed (Table I), although reduction of *O.* cf. *chalybea* numbers relative to fishless controls approached signifigance (0.1 level). Bighead carp averaged 0.65 kg, with stocking at 3 g/L per pool in triplicate. Further evaluation into algal gut contents and viability were conducted. *Oscillatoria* were viable, although fragmentation was noted.

**Table I. Bighead Carp Effects on Off-Flavor Algae**

| *Parameters* | *100-h Mean* | *% Change* | t-test *(P)* |
|---|---|---|---|
| *O.* cf. *chalybea* | 0.3 fil/ml | -92.5 | 0.1 |
| *Anabaena spp.* | 98.6 fil/ml | +124.8 | 0.3 |
| Chlorophyll *a* | 558.0 µg/L | +318.0 | 0.3 |
| Pheophytin *a* | 115.6 µg/L | +545.3 | 0.2 |

Gizzard shad *Dorosoma cepedianum* were planned to be evaluated in a similar manner, however a study (*17*) at the time found stimulation of *O.* cf. *chalybea.* This *chalybea.* This study and other reports were used to eliminate gizzard shad from further consideration

### *Bighead and Silver Carp Hybrids*

Hybrids of bighead carp and silver carp *H. molitrix* were similarly evaluated in the outdoor pools (Tables II and III). The size of the hybrids was smaller than that of bighead carp (approximately 25 g each) and pond phytoplankton samples did not include *O.* cf. *chalybea.* No significant effects were noted. However, time of the trial was 48 h and stocking was 0.1g/L. The slight (non-significant) decrease in chlorophyll *a* levels appears to reflect the increase seen with bighead carp and the significant decrease seen with silver carp in later trials. Digestion of *Oscillatoria chlorina* was not effective, as the filaments placed in water after removal from the gut began the characteristic oscillating movements.
Similar effects were seen with the silver X bighead carp cross (Table III).

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**Table II. Bighead X Silver Carp Effects on Off-Flavor Algae**

| *Parameters* | *48-h Mean* | *% Change* | *t*-Test *(P)* |
|---|---|---|---|
| *Anabaena spp.* | 0.0 fil/ml | -100.0 | 0.3 |
| Chlorophyll *a* | 201.5 μg/L | -21.5 | >0.5 |
| Pheophytin *a* | 41.8 μg/L | +197.2 | >0.5 |

**Table III. Silver X Bighead Carp Effects on Off-Flavor Algae**

| *Parameters* | *48-h Mean* | *% Change* | *t-Test (P)* |
|---|---|---|---|
| *Anabaena* spp. | 0.3 fil/ml | -92.5 | 0.3 |
| Chlorophyll *a* | 220.3 μg/L | -14.0 | >0.5 |
| Pheophytin *a* | 42.3 μg/L | +100.0 | 0.5 |

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### *Nile and Blue Tilapia and Silver Carp*

In early September, pond water containing both geosmin and MIB-producing cyanobacteria were presented to Nile tilapia, blue tilapia and silver carp in triplicate mesocosm pools. The average sizes were: Nile tilapia-155 g, blue tilapia-46 g, and silver carp-100 g. A stocking rate of 454 g per pool was used for each species or approximately 1.0 g/L (10,000 kg/ha). Comparisons with controls were made after 48 h. Fish were not fed during the trial. Zooplankton samples were also taken after 48 h.

Nile tilapia were very effective in consuming phytoplankton, including the off-flavor cyanobacteria (Table IV). The increase in pheophytin *a* is judged to indicate breakdown of chlorophyll *a* during digestion. No increases in the larger zooplankton groups were noted to account for the pheophytin *a* increase. Rather than assuming a decrease in chlorophyll *a* will occur, as indicated in the short term, the long term effect is certain to be no change or perhaps a slight increase in chlorophyll *a* levels (*18*). Small and resistant (mucilage-covered) algae have become dominant in blue tilapia ponds (*17, 19*).

**Table IV. Nile Tilapia Effects on Off-Flavor Algae**

| *Parameters* | *48-h Mean* | *% Change* | *t-Test (P)* |
|---|---|---|---|
| *O.* cf. *chalybea* | 0.0 fil/ml | -100.0 | 0.000009 |
| *Anabaena spp.* | 12.3 fil/ml | -92.9 | 0.02 |
| Chlorophyll *a* | 114.1 μg/L | -40.0 | 0.03 |
| Pheophytin *a* | 14.8 μg/L | +2114.0 | 0.002 |

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Nile tilapia zooplankton consumption was substantial: 90% decrease in cladocerans, 92% in copepods, and 86% in rotifers relative to the fish-less controls after 48 h. Nauplii were stimulated to a substantial degree (278%) however.

Silver carp and blue tilapia were next and similar in effectiveness in filtering of the target algae (Tables V and VI), as judged by the level of signifigance of the changes relative to the control. Reduction in chlorophyll *a* appears similar in silver carp to Nile tilapia. Pheophytin *a* increase was similar between blue tilapia and silver carp and about half of the Nile tilapia effect.

Silver carp reduced cladocerans to a greater degree than the tilapias (95% vs 90%), but reduced rotifers by less (75% vs 85%). Adult copepods were reduced by silver carp to a similar degree to Nile tilapia (92-93%), and nauplii were stimulated similarly to blue tilapia (388% vs 383%).

**Table V. Silver Carp Effects on Off-Flavor Algae**

| *Parameters* | *48-h Mean* | *% Change* | *t-Test (P)* |
|---|---|---|---|
| *O.* cf. *chalybea* | 24.0 fil/ml | -89.5 | 0.0004 |
| *Anabaena spp.* | 0.0 fil/ml | -100.0 | 0.01 |
| Chlorophyll *a* | 89.0 μg/L | -53.5 | 0.04 |
| Pheophytin *a* | 7.0 μg/L | +1000.0 | 0.09 |

Blue tilapia are assumed to be quite similar to Nile tilapia in their effects, although they are known to be less dependent on phytoplankton and compensate with greater consumption of detritus and zooplankton (*20*). The reduced pheophytin *a* increase and lower chlorophyll *a* decrease compared to Nile tilapia may be indicative of this (Table VI).

Blue tilapia reduced rotifers to a similar degree to Nile tilapia (85 and 86% respectively) and greater than by silver carp. More copepods were consumed by blue tilapia than Nile (96% vs 92%). As mentioned, 5% fewer cladocerans were consumed by the tilapias than silver carp. Nauplii in the presence of blue tilapia were increased to a greater extent than found with Nile tilapia (383% vs 278%), and similar to silver carp.

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**TableVI. Blue Tilapia Effects on Off-Flavor Algae**

| *Parameters* | *48-h Mean* | *% Change* | *t-Test (P)* |
|---|---|---|---|
| *O.* cf. *chalybea* | 12.7 fil/ml | -94.5 | 0.0003 |
| *Anabaena spp.* | 0.0 fil/ml | -100.0 | 0.01 |
| Chlorophyll *a* | 136.8 µg/L | -28.3 | 0.15 |
| Pheophytin *a* | 10.8 ug/L | + 1542.0 | 0.04 |

### *Threadfin Shad and Unionid Clams*

Local FFM's were tested on September 11, and unfortunately the pond algae community used in the prior evaluations changed in the loss of *O.* cf. *chalybea*. *O.* cf. *chalybea* was spiked to provide evaluation of this important off-flavor cyanobacteria species. The addition of the *O.* cf. *chalybea* led to large variations in controls and thus an inability to produce significant determinations for this alga. Zooplankton were sampled as in the preceding trial. Stocking density was 454 g per pool again or 10,000 kg/ha. Sizes were: threadfin shad-15 g, lilliput clams-20 g, and giant floater clams-140 g. Lilliput clams are *Toxolasma* sp. and giant floater clams are *Anadonta grandis*. These clams were found in and adapt to production ponds.

Threadfin shad significantly reduced *Anabaena* off-flavor algae, but not *O.* cf. *chalybea* (Table VII). As indicated, the variation in control counts of *O.* cf. *chalybea* may not have allowed significant determinations. However, the pond serving as the holding pond for threadfin shad at the time of the study developed a heavy *O.* cf. *chalybea* bloom/scum and MIB odor. The stimulation in chlorophyll *a* was highly significant and dramatic. No effect on pheophytin *a* was seen, indicating little utilization of algae. All zooplankton were impacted with pronounced reductions in nauplii (96%) and copepods (93%), followed by rotifers and cladocerans (77% each).

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**Table VII. Threadfin Shad Effects on Off-Flavor Algae**

| *Parameters* | *48-h Mean* | *% Change* | *t-Test (P)* |
|---|---|---|---|
| *O.* cf. *chalybea* | 0.0 fil/ml | -100.0 | 0.4 |
| *Anabaena spp.* | 75.3 fi/ml | -8 .0 | 0.01 |
| Chlorophyll *a* | 288.4 μg/L | +419.0 | 0.000006 |
| Pheophytin *a* | 0.0 μg/L | +/- 0.0 | - |

The two clam species did not significantly impact any of the parameters measured and thus results are not presented. Upon draining of the pools, areas surrounding each shell were cleared, indicating consumption of sedimented organic matter. No significant impacts were detected in any of the zooplankton groups.

## Experimental 0.04-0.1-ha Pond Evaluations of Promising Species

The promising species from the mesocosm studies were: Nile and blue tilapias and silver carp. Threadfin shad were effective for *Anabaena* off-flavor control, but were assumed not to be effective for *O* cf. *chalybea* (from holding pond observations). In addition, the stimulation in chlorophyll *a* was judged to be an adverse and undesirable impact. The three species were evaluated with channel catfish in 0.4-0.1-ha experimental ponds on the Aquaculture Research Station of UAPB. Systems were designed to minimize or eliminate sorting of fish species biological control is availability of a monosex Nile tilapia strain, known as the GMT or genetically male tilapia. This will be of importance if tilapia escape into the pond or from the pond. Reduced or no reproduction will occur, which is a major constraint in the use of tilapia from a culture and environmental standpoint. GMT blue tilapia could be produced if demanded.

### *Nile Tilapia*

A mixed-sex Nile tilapia polyculture system with channel catfish had been investigated before the mesocosm trials (*21*). They were effective at 5,000/ha in cages in 0.04-ha earthen ponds with channel catfish in controlling "spiked" off-flavor cyanobacteria. One month was required for the tilapia to reduce or eliminate the problem algae. Informal taste tests of catfish reflected reduced MIB. Growth of the mixed-sex tilapia resulted in an average of 0.23 kg fish without feed (limited amounts of the floating pellets were impinged aganist the cage and consumed).

A study was conducted to evaluate the effect of reducing the stocking of tilapia on off-flavor algae and water quality. The pond stocking rates of Nile tilapia in cages were 5,000/ha, 2,500/ha and 1,250/ha, each replicated three times in 0.04-ha ponds stocked with 15,000 channel catfish/ha. In the stocking rate of 5,000/ha "spiked" off-flavor algae were no longer present after an average of 29 days, in the 2500/ha stocking level the off-flavor algae were eliminated after an average of 49 days, and in the 1,250/ha stocking level after an average of 73.5 days (which may represent natural succession in October). The growth of the tilapia decreased with increasing stocking levels in cages.

## Nile Tilapia Net-Pen Evaluation in 1-ha Ponds

From July to November, a Nile tilapia net-pen study was conducted in three 1-ha earthen fingerling blue catfish (*I. Furcatus*) ponds on the Joe Hogan State Fish Hatchery, Lonoke, Arkansas (*21*). Approximately 25,000/ha 50-g blue catfish fingerlings were stocked on July 11 into all ponds. On August 11, four net pens were placed in each of two ponds and stocked with 5,000/ha 7.5-g Nile tilapia. One of the nets in each pond was stocked with the GMT tilapia, and the other nets were stocked with mixed-sex tilapia. Blue catfish fingerlings were fed a fixed percent body weight daily of 32% protein floating catfish pellets and tilapia were not fed. Water with off-flavor cyanobacteria communities dominated by *O.* cf.

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*chlaybea* were "spiked" in all three ponds on July 21. Off-flavor species of *Anabaena* were already present in substantial numbers in the ponds. Weekly water quality sampling was conducted until harvest of tilapia net pens on November 10. *O.* cf. *chalybea* were eliminated in one month in one pond and reduced to 100 intact filaments/ml in the other polyculture pond. The monoculture pond contained 1,300 intact filaments/ml at this time. Informal taste tests confirmed the greater MIB levels. The percentage of weeks with numbers approximating or in excess of 1,000 filaments/ml *O.* cf. *chalybea* were 67% in the monoculture pond and 0% in the polyculture ponds. The percentage of weeks with numbers approximating or in excess of 1,000 intact filaments/ml of off-flavor *Anabaena* were 33% in monoculture and 0% in polyculture. Problem cyanobacteria levels of 1000-2000 natural units/ml have been associated with taste and odor problems in Australia (*22*). Chlorophyll *a* levels were not significantly different between ponds.

The inability of the tilapia system to eliminate the off-flavor algae, unlike the earlier tests in smaller ponds, was reflected in the slight remaining off-flavors in the informal taste tests. Algae numbers and chlorophyll *a* concentrations were higher than in the 0.04-0.1-ha trials. In addition, the size of the cage relative to the pond surface area was smaller. Perhaps circulation will be required for the net pens. Unfed tilapia grew from 7.5 g to an average of 0.07 kg in one pond and 0.1 kg in the other. This is approximately 1 g/d growth. Over the normal growing season of 300 days, the final average weight would have been expected to be 0.21 kg and 0.3kg, respectively. GMT tilapia were 25% larger than mixed-sex tilapia.

## Conclusions

Although promising, filter-feeding fishes as biological controls of off-flavor cyanobacteria require further evaluation on a commercial scale. Measurement of MIB and geosmin levels with algae abundances is needed to determine levels allowing on-flavor designation and confirm or refute the 1000 filaments/ml threshold. Threadfin shad should be tested again to determine effects on *O.* cf. *chalybea,* the major off-flavor alga. These systems should also be tested with other cultured species experiencing off-flavors, which would differ in levels of intensity, water sources and system design and engineering. The cost of the FFM system and the potential of the system to pay for itself through marketable filter-fishes should be investigated through cost/benefit analyses. GMT tilapias offer significant advantages in growth characteristics compared to mixed-sex (*23*), and do not have the potential for environmental impacts through establishment after escape.

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## Chapter 13

# Secondary Metabolites from Plants and Marine Organisms as Selective Anti-Cyanobacterial Agents

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**[2]Natural Products Utilization Research Service, National Center for Natural Products Research, Agricultural Research Service, U.S. Department of Agriculture, University, MS 38677–8048**

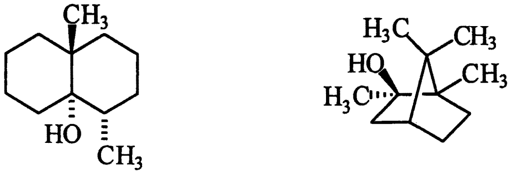
Extracts of more than one thousand species of plants and marine organisms were evaluated for selective algicidal activity against the musty-odor cyanobacterium (blue-green alga) *Oscillatoria perornata.* Bioassay-guided fractionation yielded anti-cyanobacterial compounds from the tropical marine brown alga *Dictyota dichotoma.* The structures of the active metabolites were confirmed spectroscopically to be two bicyclic diterpenes 10-acetoxy-18-hydroxy-2,7-dolabelladiene and dictyol B acetate.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Aquaculture of commercially important fish and other seafood products has become a vital component of agriculture and food production in the United States. However, blooms of noxious cyanobacteria (blue-green algae) in aquaculture facilities threaten this industry and may pose a potential health concern. Bloom-forming cyanobacteria produce a wide array of biologically active natural products. Some of these cyanobacterial compounds are toxic to fish. Other noxious and distasteful metabolites can accumulate in the flesh of farm-raised fish and ruin their commercial value.

Members of the freshwater cyanobacterial genera *Microcystis* and *Nodularia* produce hepatotoxic peptides known as microcystins and nodularins (*1*). Neurotoxic alkaloids produced by the cyanobacterium *Anabaena flos-aquae* target vital neuromuscular communication pathways by interfering with normal regulation of neuronal ion flux. The occurrence of these cyanotoxins from blue-green algal blooms has resulted in massive fish kills, numerous livestock poisonings, and linked to serious intoxication events in people.

Undesirable species of bloom-forming filamentous cyanobacteria produce noxious compounds that render the flesh of pond-raised fish unpalatable and of little or no commercial value (*2*). While relatively non-toxic, the accumulation of these lipophilic compounds in the fatty and muscle tissues of fish results in the condition known as "off-flavor" by the commercial fish farming industry (*2*). The cyanobacterial metabolites most associated with off-flavor are the "earthy" tasting compound geosmin [**1**] and the “musty” tasting compound 2-methylisoborneol [**2**] (Figure 1). These substances do not harm the fish. However, they seriously damage the commercial profitability of the industry, as they force fish farmers to hold unmarketable stocks for weeks or even months while waiting for these substances to be cleared from the fish tissues (*2*).



**1** **2**

*Figure 1. Cyanobacteria metabolites geosmin [1] and methylisoborneol [2] responsible for "off-flavor" in channel catfish raised in commercial fishponds.*

Farm-raised channel catfish (*Ictalurus punctatus* Rafinesque) represent a major agricultural and economic commodity in the southeastern U.S. (*3*). One of the most prevalent off-flavor producing organisms that blooms in the aquaculture ponds of western Mississippi is the filamentous cyanobacterium *Oscillatoria perornata* f. *attenuata* (Skuja) [recently reassigned as *Planktothrix perornata* f. *attenuata* (Anagnostidis and Komárek)] (*3*).

Certain cyanobacteria and algae have been reported to produce antimicrobial and herbicidal metabolites that may have allelopathic properties that control the growth of other related species (*4*). Freshwater cyanobacteria

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have been found to produce secondary metabolites that inhibit photosynthesis in other aquatic cyanobacteria and algae. The fischerellins A [**3**] and B [**4**] (from *Fischerella musciola* Thuret) (*5-8*) and cyanobacterin [**5**] (from *Scytonema hofmanni*) (*9-15*) inhibit photosystem II-mediated electron transport (Figure 2). Other antialgal substances produced by cyanobacteria include hapalindole A [**6**] (from *Hapalosiphon fontinalis*) (*16,17*), nostocyclamide [**7**] (from *Nostoc* sp.) (*18,19*), and kasumigamide [**8**] (from *Microcystis aeruginosa*) (*20*). Allelopathic fatty acids and bromophenolic compounds have been identified from macrophytic marine algae (*21-24*).

While some algal metabolites have antialgal activity, many of these compounds may be nonspecific antimicrobial agents or generally cytotoxic. The nonspecific nature of antibiotics and cytotoxic metabolites render those substances of little use as agents to control harmful algal blooms in aquaculture ponds. Perhaps the lack of anticyanobacterial specificity associated with many natural products is simply the result of the nonselective nature of the bioassay methods used for their discovery. The assay systems used in these studies did not clearly distinguish between with selective cyanobactericidal activity and those that inhibit eukaryotic algae. Much of this research has focused on the potential ecological implications of allelopathic natural products or their potential as herbicides, rather than on the discovery of anticyanobacterial algicides. Further, few studies use bioassay systems that simultaneously examine and compare efficacy in both cyanobacteria and eukaryotic algae. Cyanobacteria are photosynthetic Gram-negative bacteria. However, these studies fail to provide data as to the antibacterial activity of anticyanobacterial natural products against other non-photosynthetic species of bacteria. Before any antialgal material can be considered selective enough for use in aquaculture, it must be determined to be nontoxic towards fish, humans, and the environment. Therefore, anticyanobacterial compounds with significant toxicity to either fish or mammals cannot be considered safe for development as a means to control blue-green algal blooms in fish farming ponds.

Crude natural product-rich extracts and purified compounds were evaluated for cyanobacteria-selective antialgal activity using a recently developed high-throughput method (*25-27*). This bioassay system simultaneously examines natural products for antialgal activity against the off-flavor producing cyanobacterium *Oscillatoria perornata* and the unicellular green alga *Selenastrum capricornutum.* An evaluation of differential cyanobacteria selectivity is achieved by identifying those crude extracts and purified compounds that, over a series of half-log concentration gradients, inhibit *O. perornata* without affecting the *S. capricornutum.* Substances found to have anticyanobacterial activity are then evaluated for antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria. Extracts and purified compounds that appear to function as non-selective antibiotics are deselected for further study. Crude extracts and purified natural products are further evaluated

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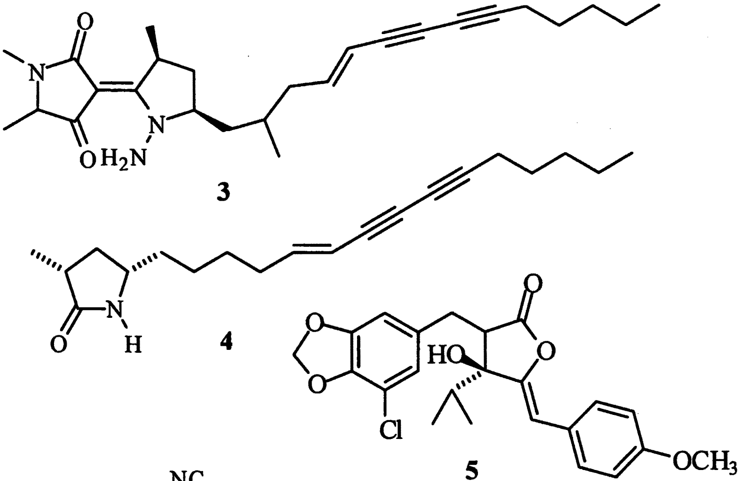
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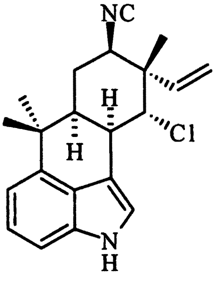

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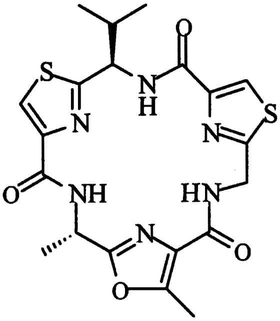
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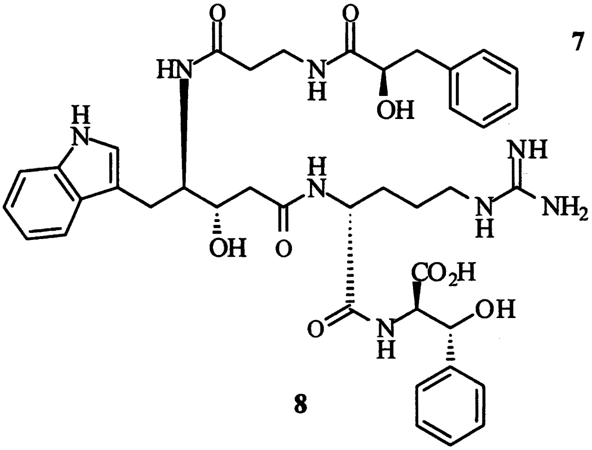
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*Figure 2. Antialgal compounds produced by cyanobacteria.*

for toxicity to normal mammalian cells using a standardized Vero Cell cytotoxicity assay. Substances found to be cytotoxic are deselected for further development.

Spurred by indications from prior allelopathic studies, we have examined the extracts of more than one thousand species of cyanobacteria, algae, and higher plants for cyanobacteria-selective antialgal activity. Repositories of pure compounds from marine algae and invertebrates were also examined. Those found to be non-selective or cytotoxic were not further studied. Bioassay-guided fractionation and purification has resulted in the isolation and identification of anticyanobacterial natural products. This chapter discusses ongoing research and anticyanobacterial bioassay "hit rates," as a function of the extract source organism. The chemical structures and cyanobacteria-selective antialgal activities of two brown algal diterpenes (from *Dictyota menstrualis)*, identified through bioassay-guided evaluation of marine algae extracts, are presented.

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## Experimental Section

### Sample Acquisition and Preparation

Internationally-collected terrestrial tropical plant samples (from Peru and New Guinea, National Center for Natural Products Research [NCNPR] collection) were dried in a plant dryer (at 38° C) and ground to a powder before extraction. Marine sponges, cyanobacteria, and algae (collected mainly from Indonesia, Jamaica, Guam, and the United States) and plants collected in the southern U.S. were lyophilized prior to solvent extraction. The sample of *Dictyota menstrualis* (Sample ID#: DNJ.040) that provided the anticyanobacterial diterpenes **12** and **13** was hand collected from Discovery Bay, Jamaica at a depth of -1M in July 1998.

Crude ethanolic extracts of terrestrial plants from Peru, New Guinea, and the United States (NCNPR collection) were prepared by extracting dried and ground plant material with 95% ethanol (EtOH). Lipophilic extracts of freshwater and marine organisms were prepared by extracting lyophilized cyanobacteria, algae, and aquatic plants with a 2:1 solution of methylene chloride ($CH_2Cl_2$) in methanol (3X). Aquatic and marine specimens were then further extracted with 100% methanol (MeOH) to prepare an additional extract of intermediate polarity. Crude extracts were dried under vacuum, weighed, and used to prepare stock solutions for bioassays. In general, crude extract stock solutions were prepared in dimethyl sulfoxide (DMSO) at 2.0 mg/mL and placed in the appropriate wells of 96-well microtiter plates for high-throughput bioassay. Stock solutions of certain freshwater and marine organisms were prepared in 95% EtOH for evaluation. Solvents were allowed to evaporate completely from microplate wells before adding cyanobacterial and algal culture material.

Purified marine invertebrate compounds were obtained as a consequence of other research projects in the Department of Pharmacognosy at the University of Mississippi. The chemical structures and pharmacological properties of most of these substances were the subject of previously published research. These substances were prepared in DMSO stock solutions at 2.0 mg/mL, and solutions were placed in the wells of 96-well microtiter plates for high-throughput bioassay.

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## Extraction and Isolation of Antialgal *Dictyota menstrualis* Metabolites

The extraction of *Dictyota menstrualis* material was scaled-up to produce sufficient crude extract for chromatographic separation and biological evaluation of antialgal constituents. Lyophilized *D. menstrualis* material (42.79 g) was extracted (4X) with 2:1 $CH_2Cl_2$:MeOH at room temperature. The solvent was evaporated under reduced pressure to give 1.68 g of crude extract (19.7 g residual extracted dry marc).

A portion (1.00 g) of the crude hexanes fraction was fractionated by vacumm liquid chromatography (VLC) using Hexanes:EtOH step-gradient, and 14 fractions were obtained. The fraction most active in the bioassay (fraction #3; 212.9 mg) was then further separated on ODS-SPE cartridge using the following: I) 90% MeOH in $H_2O$ (v/v); II) 95% MeOH in $H_2O$ (v/v); and III) 100% MeOH, in a stepwise gradient to obtain three fractions. Fractions #1 and #2 formed clear crystals during evaporation. The crystals were separated from the mother liquor. The active mother liquor from fraction #1 was chromatographically separated by Si-gel open column using a gradient of (0-0.9% 2-propanol in hexanes) and the combined non-polar fractions were chromatographed by normal phase-HPLC [Econosil, 10 μM, 250 mm x 22 mm, 5% isopropanol in hexanes (v/v)] to yield five fractions. Fraction #4 (38 mg) was further separated by Sephadex LH-20 size-exclusion chromatography [50% $CH_2Cl_2$ in MeOH (v/v)]. Antialgal activity of the eluted fractions was tested. Two active fractions were separated by HPLC [Econosil $C_{18}$ 10 μM, 22 mm x 250 mm, 10% $H_2O$ in MeOH (v/v)] to provide compound **12** (0.8 mg, 0.08% yield) and **13** (5.1 mg, 0.51% yield). The remaining fractions were found to be inactive.

## Antialgal Bioassays

Crude extracts and purified compounds were evaluated for antialgal activity using a rapid, reproducible 96-well microtiter plate bioassay (*25*). The cyanobacterium *Oscillatoria perornata* f. *attenuata* (Skuja) and unicellular chlorophyte *Selenastrum capricornutum* were grown in continuous, steady-state cultures to provide cells growing at a constant rate. Stock solutions of extracts, chromatographic fractions, and purified compounds were tested by adding the stock solutions (2.0 μl) in duplicate to the wells in 96-well culture microplates. Solvents were allowed to completely dry (DMSO was completely dried under vacuum) before 200 μL of algal and cyanobacterial culture material was added to appropriate wells. Culture was removed aseptically from the continuous culture systems and micropipetted into the wells of 96-well microplates (200 μL/well). Microplates were placed in growth chambers (Percival Scientific model I-36LL) at the same light intensity and temperature used to maintain the continuous culture systems. Specifically, 28-30°C and illumination by overhead fluorescent lamps (20W) at a light intensity of 18-24 $\mu E/m^2/sec$. The absorbance (650 nm) of each well was measured daily for a minimum of three days using a Packard SpectraCount microplate photometer. Initially, crude extracts were evaluated in duplicate using a high-throughput single concentration method. The antialgal bioassay was first performed using prepared solutions of lipophilic crude extracts in modified BG-11 (Izaguirre) growth medium at 100 μg/mL. Extracts found to be antialgal were screened at half-log concentration increments of 30 μg/mL and 10 μg/mL. In the first phase of screening, 356 extracts of freshwater and marine cyanobacteria, algae, and wetland/aquatic plants were evaluated. In the second phase of screening, 30 extracts were tested.

For purified compounds, a series of concentrations were evaluated (e.g. 10, 5.0, and 1.0 μg/mL). Absorbance readings for each concentration were graphed and calculations done to extrapolate an $IC_{50}$ and determine a LOEC (lowest-observed-effect concentration) and a LCIC (lowest-complete-inhibition concentration). The relative toxicity and selectivity of crude extracts and purified compounds were determined by comparing graphed data for *O. perornata* and *S. capricornutum*.

## Evaluation of Purified *Dictyota menstrualis* Diterpenes

Compounds **12** and **13** (Figure 5) were dissolved in 95% EtOH to make final stock solutions of 2 mg/mL. Culture material was added to wells of 96-well microplates (200 μg/well), and three concentrations (1.0, 5.0, and 10 μg/mL final concentrations in wells) of each compound were tested. Three replicates of each concentration of each compound and controls were used. Mean values of absorbance measurements for each concentration and controls were graphed. The mean values of algal and cyanobacterial growth inhibition were calculated from 0 to 72 h ($IC_{50}$). Compounds **12** and **13** inhibited *Oscillatoria perornata* with $IC_{50}$ = 23.4 μM and $IC_{50}$ = 2.23 μM, respectively. Neither compound showed any inhibition of *S. capricornutum* at any concentration tested.

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Absorbance readings can be affected by the settling of dead cyanobacterial cells in the microplate well bottoms, thereby artificially elevating the apparent $IC_{50}$ of antialgal compounds. Therefore, these assays were also inspected visually. Visual inspection of the *O. perornata* treated with **12** and **13** confirmed the growth inhibition absorbance measurements. Visual observation of the wells also indicated that **12** and **13** completely kill *O. perornata* at 5.0 µg/mL (14.5 µM). However, neither **12** nor **13** significantly inhibit *O. perornata* at 0.5 µg/mL.

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## Antibacterial Bioassays

The antibacterial bioassays used are adaptations of those recommended by the National Committee on Clinical Laboratory Standards (*28*). Inocula of bacteria [*Staphylococcus aureus* ATCC 29213, Methicillin-resistant *S. aureus* ATCC 43300 (MRS), and *Pseudomonas aeruginosa* ATCC 2785] were prepared by diluting 24-h cultures in Eugon nutrient broth supplemented with OADC (oleic acid-albumin-detrose-catalase). Crude extracts and column fractions were added to the wells of a 96-well microtiter plate at final concentrations of 20, 100, and 500 µg/mL while pure compounds at 0.20, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 µg/mL. Inocula of the appropriate organism were added such that the final volume in each well was 200 µg/mL. Ciprofloxacin (ICN Biomedicals, Ohio) was used as a positive control. Following standard incubations periods of 18-24 h at 37°C, the inhibitory activity of crude extracts was extrapolated to obtain an $IC_{50}$ value. Inhibitory activity of purified compounds was assessed as the Minimum Inhibitory Concentration (MIC) (the lowest test concentration in which no detectible growth is observed). Inhibitory activity was assessed by measurement of turbidity at 630 nm using an EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont).

## Mammalian Cytotoxicity Assay

Crude extracts and purified compounds were evaluated for cytotoxicity toward mammalian cells using VERO cells as a standardized *in vitro* model. VERO cells are non-cancerous cells derived from the kidney of a normal adult African green monkey [obtained from the American Type Culture Collection, Manassas, Virginia (ATCC)]. This cell line has been widely used to detect toxins and viruses. VERO cell viability was measured using the Neutral Red Cytotoxicity method (*29*). VERO cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified atmosphere (5% $CO_2$ and 95% air) at 37°C. Exponentially-grown VERO cells were transferred into 96-well culture plates. The cells were incubated for 18 h and then crude extracts and purified compounds (in stock solutions) were added at three concentrations. Equal volumes of solvent were added as controls. After incubation at 37°C for 48 h, the conditioned medium was removed and culture medium that contained 0.15 mg/mL neutral red was added to the wells. After incubation at 37°C for two hours, the medium was removed, the wells were washed once with saline solution (0.9%), and the cells were lysed with an acidified solvent (0.04N HCl in isopropanol). Absorbance (A) (540 nm) was measured, and background absorbance (630 nm) was subtracted. Only viable cells will pick up neutral red from the medium, and the $A_{540}$ value correlates with the level of cell viability. Only antialgal extracts and compounds that were found to have no significant cytotoxicity to VERO cells were pursued further.

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## Spectroscopic and Spectrometric Methods Used to Determine Structures of Anticyanobacterial Natural Products

$^1$H and $^{13}$C NMR spectra of **12** and **13** were recorded in $CDCl_3$ on a Bruker DRX 400 spectrometer operating at 400 MHz for $^1$H, and 125 MHz for $^{13}$C, by running gradients, and by using residual solvent peaks as internal references. The HREIMS data were acquired on Bruker BioAPEX 30es.

Optical rotations were measured on a Perkin-Elmer 343 polarimeter. The IR spectrum was obtained using an AATI Mattson Genesis™ Series FTIR, and UV spectra were obtained using a Hewlett-Packard 8453 Spectrophotometer.

## Data for Purified *Dictyota menstrualis* Diterpenes

***Compound [12]: 10-acetoxy-18-hydroxy-2,7-dolabelladiene.*** Clear oil. $[\alpha]_D^{25}$ = -40.9 (c = 0.06, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 208 (3.34) nm; IR (film) $\nu_{max}$ 3449, 2955, 2947, 2850, 1736, 1237, 1111, 1018 cm$^{-1}$; $^1$H NMR ($CDCl_3$) $\delta$ 0.88 (3H, s, $H_3$-15), 0.95 (3H, d, $J$ = 6.8 Hz, $H_3$-16), 1.19 (3H, s, $H_3$-20), 1.27 (3H, s, $H_3$-19), 1.43 (2H, m), 1.56 (2H, m), 1.65 (3H, s, $H_3$-17), 1.77 (1H, dd, $J$ = 10.2, 2.0 Hz, H-11), 1.88 (1H, m), 2.01 (2H, m), 2.07 (3H, s, $H_3$-22), 2.15 (2H, m), 2.34 (2H, m), 4.82 (1H, td, $J$ = 11.0, 2.5 Hz, H-10.) 5.09 (1H,d, $J$ = 16.0 Hz, H-2), 5.13 (1H, d, $J$ = 7.8 Hz, H-7), 5.25 (1H, dd, $J$ = 16.0, 7.4 Hz, H-3), 5.35 (1H, br. s, OH); $^{13}$C NMR ($CDCl_3$, 125 MHz.) $\delta$ 18.51(C-17), 19.79 (C-15), 21.65 (C-16), 21.89 (C-22), 23.19 (C-20), 27.09 (C-6$^*$), 30.14 (C-13$^*$), 32.85 (C-19), 36.14 (C-5), 38.02 (C-4), 39.69 (C-14), 45.72 (C-9), 47.29 (C-1), 49.61 (C-12), 55.83 (C-11), 72.27 (C-18), 73.38 (C-18), 127.67 (C-8), 131.19 (C-7), 134.94 (C-2), 135.46 (C-3), 169.46 (C-21); HREIMS m/z: 371.254 (M+Na)$^+$; for $C_{22}H_{36}O_3Na$ calc 371.255. ($^*$interchangeable)

***Compound [13]: dictyol B acetate.*** Clear oil. $[\alpha]_D^{25}$ = +91.75 (c = 0.4, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 216 (3.16) nm; IR (film) $\nu_{max}$ 3527, 2960, 2917, 2855, 1725, 1373, 1241, 896 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$1.02 (3H, d, $J$ = 6.4 Hz, $H_3$-19), 1.24 (1H, m, $H_2$-12), 1.60 (3H, s, $H_3$-20), 1.62 (1H, m, H-7), 1.69 (3H, s, $H_3$-16), 1.82 (3H, s, $H_3$-17), 1.96 (1H, m, $H_2$-13), 2.15 (3H, s, $H_3$-22), 2.26 (1H, m, $H_2$-2), 2.36 (1H, m, H-5), 2.58 (2H, m, H-2, H-1), 3.92 (1H, dd, $J = 7.63, 3.9$ Hz, H-6), 4.93 (1H, s, H-18), 4.97 (1H, s, H-18), 5.11 (1H, d, $J = 3.1$ Hz, H-14), 5.15 (1H, dd, H-9), 5.34 (1H, s, H-3); $^{13}$C NMR ($CDCl_3$, 125 MHz) $\delta$15.82 (C-17), 17.59 (C-19), 17.92 (C-20), 21.49 (C-22), 25.76 (C-13), 25.92 (C-16), 30.40 (C-7), 33.88 (C-2), 34.90(C-11), 35.13 (C-12), 43.20 (C-1), 44.05 (C-8), 61.21 (C-5), 74.70 (C-6), 77.50 (C-9), 104.92 (C-18), 124.13 (C-3), 124.70 (C-14), 131.96 (C-15), 141.00 (C-4), 149.69 (C-10), 170.25 (C-21); HRESIMS m/z 369.2371 $(M+Na)^+$ for $C_{22}H_{34}O_3Na$ calc. 369.2400.

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## Results and Discussion

### Terrestrial plants

Initial evaluation of plant extracts at 100 µg/mL revealed that many plants contained relatively high concentrations of tannins that had weakly potent antimicrobial activity with little or no cyanobacterial specificity. Thus, these tannins acted as bioassay "nuisance" compounds and provided an unacceptably high false-positive hit rate, thereby precluding the rapid identification of potent and selective substances. Therefore, this method was modified to accommodate the high-throughput evaluation of hundreds of crude extracts, chromatographic fractions, and pure natural product samples prepared in a 96-well plate format. Subsequent evaluation of crude extracts was then performed in duplicate at 20 µg/mL, and extracts found to have antialgal activity were retested at 10 and 2.0 µg/mL. These modifications increased evaluation efficiency and dramatically reduced the numbers of antialgal "hits" attributed to tannins and other non-selective antimicrobial substances.

A biogenetically diverse repository of plant extracts from over 170 plant families found in tropical rainforest and temperate regions throughout the world were evaluated. These plants were obtained mainly in Peru, New Guinea, and the United States. This repository contained chemically distinct, crude lipophilic extracts of separate plant parts (roots, leaves and stems, flowers, etc.) of each species collected. Over 2300 crude extracts of more than 1050 species of higher plants were evaluated.

Over 70 plant species showed some level of selective activity against *O. perornata*. As mentioned earlier, the major goal of this research program was to identify anticyanobacterial specific agents. Therefore, the active extracts that showed broad antimicrobial activity against a panel of biomedically important microorganisms were considered to be "nonspecifically antibacterial" and were deselected for further study.

The most potent cyanobacteria-selective terrestrial plant extracts (that were not significantly antibacterial and were non-cytotoxic) were those obtained from the roots and stems of a Peruvian collection of *Dulacia candida* (Olacaceae). *Dulacia candida* is a widely distributed tropical Amazonian plant that grows as a shrub or small tree. Crude *D. candida* extracts showed extremely potent anticyanobacterial activity at low concentrations approaching the parts per billion range. The antialgal activity was confined to several relatively polar chromatographic fractions of the *D. candida* extract. However, the identity of the active constituent(s) has not been resolved.

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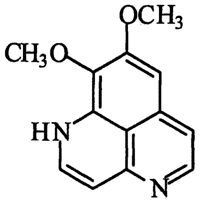
## Marine Invertebrates

Dozens of biologically-active pure secondary metabolites produced by sponges, gorgonian soft corals, and other marine invertebrates were evaluated for selective anticyanobacterial activity in this study. From this repository of pure marine natural products, the most potent and selective invertebrate metabolite found was the sponge alkaloid known as aaptamine [9] (Figure 3). Aaptamine and related alkaloids are produced by the marine sponges *Aaptos aaptos* and *Luffariella* sp. (*30,31*).



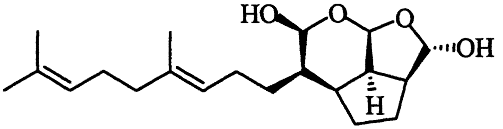
**9**

*Figure 3. Structure of aaptamine [9] from marine sponge Aaptos aaptos.*

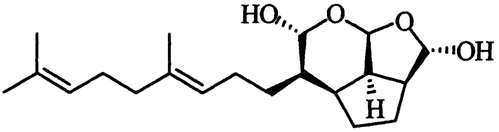
Aaptamine [9] was cyanobactericidal to *O. perornata* at 1.0 μg/mL (4.4 μM). Numerous studies have shown 9 and related metabolites to be potent and cytotoxic to mammalian cells (*32*). Due to the rather non-selective cytotoxicity of aaptamine and aaptamine analogs, these compounds were deselected for further development.

## Tropical Marine Algae

The bioassay system worked quite well for the evaluation of extracts prepared from aquatic and marine organisms. In the first phase of the project, 356 extracts from collections of aquatic and marine cyanobacteria, plants, and algae were evaluated. Forty-three extracts (12%) were found to be strongly active and cyanobacteria-selective at the concentration of 100 μg/mL. Dose response data was obtained for these active extracts at half-log concentrations. Twelve extracts were found to be effective at 30 μg/mL or lower. The two most potent extracts were confirmed to be from two samples of the tropical marine green alga *Udotea flabellum* (Ellis and Solander) Lamouroux. Bioassay-guided fractionation of the active substances from *U. flabellum* yielded several previously isolated unusual diterpenes known as udoteatrial hydrates [**10**] and [**11**] (Figure 4) (*33-35*). The structures of these substances were determined from detailed analysis of $^1$H, $^{13}$C, and 2D-NMR spectra. Along with the previously identified udoteatriols, two analogous MeOH-trapped solvolysis products were found to be effective against *O. perornata* at concentrations in the parts per billion range (less than 0.1 μg/mL). However, these *Udotea* terpenes were not significantly cyanobacteria-selective and showed antibacterial activity. Therefore, they were deselected for further evaluation.



**10**



**11**

*Figure 4. Structures of udoteatrial hydrates [10] and [11] from the marine chlorophyte Udotea flabellum.*

The crude lipophilic extract of the Caribbean brown alga *Dictyota menstrualis* Hoyt (formerly *Dictyota dichotoma* Hudson) (Dictyotaceae) completely and selectively inhibited *O. perornata* in the antialgal assay without affecting the green algal control at both 30 and 100 μg/mL. Bioassay-guided chromatographic fractionation of the crude extract yielded two cyanobacteria-selective substances. The chemical structures of these compounds were determined by a combination of high-field NMR and mass spectrometric data. These antialgal compounds were determined to be two previously isolated *Dictyota* diterpenes, 10-acetoxy-18-hydroxy-2,7-dolabelladiene [**12**] and dictyol B acetate [**13**]. The spectral data of **12** was matched with the data published for the known compound 10-acetoxy-18-hydroxy-2,7-dolabelladiene (*36,37*). The NMR spectra of **13** was in good agreement with that of dictyol B acetate (*38*)

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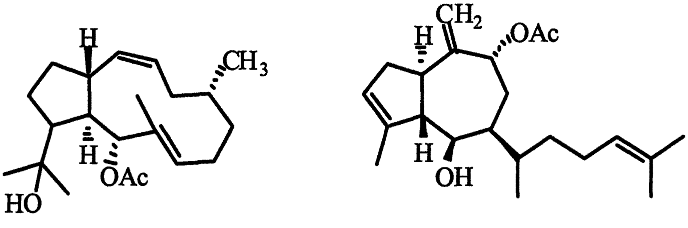
and the original chemical shifts were confirmed. However, several chemical shifts were reassigned using a combination of high-field 2D-NMR data ($^1H$-$^1H$ COSY, DEPT, HMBC, HMQC, and NOESY). The structures were additionally confirmed by determination of the chemical formula from the high-resolution ESI-Mass Spectrum and optical rotations consistent with the absolute structures of both previously published compounds.



**12** **13**

*Figure 5. Structures of cyanobacteria-selective antialgal diterpenes 10-acetoxy-18-hydroxy-2,7-dolabelladiene [12] and dictyol B acetate [13].*

Purified compounds **12** and **13** (Figure 5) were evaluated for antialgal activity and both were shown to selectively inhibit the cyanobacterium *O. perornata*. However, dictyol B acetate [13] ($IC_{50}$ = 2.23 μM) was significantly more potent than 10-acetoxy-18-hydroxy-2,7-dolabelladiene [12] ($IC_{50}$ = 23.4 μM) (Figure 6). Neither compound significantly inhibited *S. capricornutum* at the highest concentration tested, 29 μM.

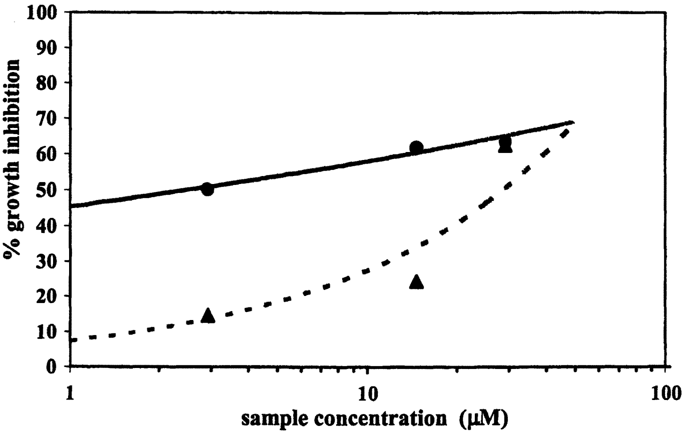


*Figure 6. Anticyanobacterial (O. perornata) dose-response curves for: ▲) 10-acetoxy-18-hydroxy-2,7-dolabelladiene [12] and ●) dictyol B acetate [13].*

Both **12** and **13** were further evaluated for antibacterial and antifungal activity in order to establish true cyanobacteria-selectivity, as opposed to non-specific antibiotic activity. Neither compound showed significant antimicrobial activity at any concentration tested (maximum concentration tested was 145 μM). Both compounds were evaluated for toxicity against mammalian cells in a VERO cell cytotoxicity assay. No cytotoxicity was observed at any concentration tested (136 μM and lower concentrations).

Various species of *Dictyota* are among the most chemically rich species of marine algae. Nearly 80 diterpenes have been isolated from over thirty species collected from different locations throughout the world (*39*). Studies of *Dictyota* spp. chemical-ecology indicate that some of these diterpenes act as deterrents to herbivore grazing (*40-43*). While several diterpenes isolated from *Dictyota menstrualis* have shown some antibacterial activity, no link has been established between *Dictyota* diterpenes and allelopathic-type activity against cyanobacteria. The assay organism *O. perornata* is a fresh water cyanobacterium, rather than marine. Therefore, truly relevant ecological conclusions can not be deduced. These *Dictyota* diterpenes are structurally unique among any other class of anticyanobacterial natural products, and their mechanism(s) of anticyanobacterial activity is unknown. However, these antialgal triterpenes may potentially serve as novel chemical prototypes for the design of new selective agents to help control harmful blue-green algae blooms in aquaculture facilities.

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## Chapter 14

# Natural Algicides for the Control of Cyanobacterial-Related Off-Flavor in Catfish Aquaculture

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Over the past two decades, a variety of natural compounds have been studied for their potential use as selective algicides against cyanobacteria to prevent musty off-flavor in cultured catfish. Some of the compounds that have been tested include unsaturated fatty acids, lysine, ferulic acid, anthraquinone, and artemisinin. In addition, decomposing barley straw has been evaluated for preventing blooms of musty-compound producing cyanobacteria in catfish ponds. So far, none of these compounds have proven to be commercially acceptable for the management of cyanobacteria blooms in Mississippi catfish ponds. However, some quinones have been found to be the most promising leads in the discovery of an environmentally-safe, natural algicide for catfish aquaculture.

Musty "off-flavor" is the most common type of flavor problem in channel catfish (*Ictalurus punctatus*) raised in Mississippi. This musty taint is due to the accumulation of the compound 2-methylisoborneol (MIB) in the flesh of the catfish. Certain types of cyanobacteria (blue-green algae) produce MIB and the cyanobacterium *Oscillatoria perornata* (Skuja) is attributed with being the major culprit for MIB-related off-flavor in west Mississippi (*1*). One management

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approach used by catfish producers for preventing musty off-flavor in catfish is the application of chemicals (algicides) to the pond in order to kill the noxious cyanobacteria. Currently, copper-based products (e.g., copper sulfate, chelated copper products) and the herbicide diuron are the only chemicals approved by the United States Environmental Protection Agency (USEPA) for use as algicides in aquaculture ponds. These compounds have several undesirable attributes including a high degree of persistence in the environment, broad-spectrum toxicity towards phytoplankton, and the public's negative perception of the use of synthetic compounds in food-fish production ponds. The discovery of environmentally-safe, natural compounds that are highly selective as algicides towards undesirable cyanobacteria in catfish production ponds would greatly benefit the catfish industry. Past and ongoing research efforts on the use of natural compounds as algicides in catfish aquaculture are discussed.

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## Anticyanobacterial Fatty Acids from *Eleocharis microcarpa*

Several fatty acids found in extracts from the aquatic plant *Eleocharis microcarpa* were reported to be antialgal and potentially useful in controlling musty off-flavor problems in farmed catfish by van Aller and Pessoney (*2*). This research is the first published record of the use of plant-derived compounds to help prevent musty off-flavor problems in farm-raised catfish. The antialgal extract fractions from *E. microcarpa* contain a mixture of oxygenated fatty acids including a novel oxygenated fatty acid (Figure 1A) (*3*). Ricinoleate (Figure 1B), linoleate, dodecanoate, and 9,10-dihydroxystearate were reported to be selectively toxic towards certain species of cyanobacteria at concentrations of 0.1 ppm to 2 ppm (*4*). Ricinoleate was found to be toxic, but not completely inhibitory, at 1 and 10 μM (approximately 0.3 and 3.0 ppm, respectively) towards *O. perornata* in laboratory microplate bioassays (*5*). Soliricin 135® (Caschem, Inc., Bayonne, NJ) is an algicide that was developed from the discovery by van Aller and Pessoney (*4*) and contains the active ingredient potassium ricinoleate (the water-soluble form of ricinoleate). This product was marketed as a selective cyanobacteriocide and granted approval by the USEPA for use in food-fish production ponds. However, in subsequent efficacy studies, the application of Soliricin 135® at levels exceeding label-recommended rates to catfish ponds revealed no reduction in numbers of cyanobacteria and did not prevent severe off-flavor in catfish from treated ponds (*6*). The researchers provide two explanations for the failure of potassium ricinoleate in these replicated pond studies: 1) a lack of persistence of the compound due to rapid metabolization by microbes; and 2) the precipitation of ricinoleate when applied to the hard water in the catfish ponds used in the studies. Soliricin 135® is no longer commercially available.

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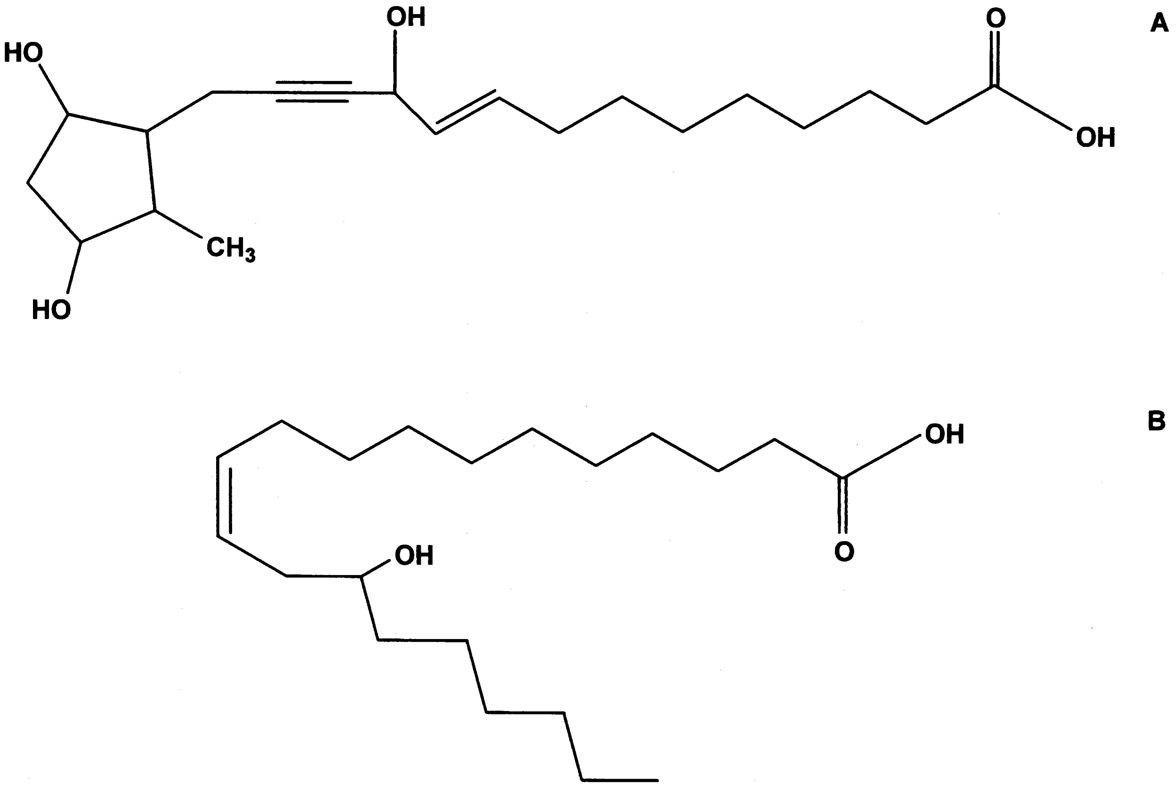

*Figure 1. Chemical structures of a novel oxygenated fatty acid (A) and ricinoleate (B).*

Table I lists the results from the screening of several fatty acids by the method of Schrader et al. (*7*) to determine their selective toxicity towards *O. perornata* with *Selenastrum capricornutum* used a representative green algae species (K.K. Schrader, previously unpublished observations). Erucic acid and linolenic acid were the most toxic towards *O. perornata*. Water-soluble salts of fatty acids found to be algicidal in laboratory studies may be relatively ineffective in killing cyanobacteria in catfish ponds due to their precipitation as insoluble salts in hard water (100 mg/L or greater as $CaCO_3$).

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## Antialgal Activity of Decomposing Barley Straw

The control of phytoplankton in surface waters by decomposing barley straw has been reported from research conducted in the United Kingdom (*8-13*). Toxic compounds released by the decomposing barley straw are believed to be responsible for this algicidal activity. The oxidation of phenolic compounds and lignin derivatives from decomposing barley straw under aerobic conditions may yield compounds, such as quinones, that are antialgal (*14*). Several naturally occurring quinones have been found to be selectively toxic towards *O. perornata* in laboratory studies (*15*). Several lignin decomposition products and intermediate compounds from the decomposition of phenolics were also screened, and syringic acid (a phenolic decomposition product) was the only one that was selectively toxic towards *O. perornata* (*15*).

Efficacy studies have been performed to determine the usefulness of decomposing barley straw to control cyanobacteria in Mississippi catfish ponds (*16*). Unfortunately, results from these studies did not detect any consistent differences in the occurrence of off-flavor in catfish from ponds treated with decomposing barley straw and in catfish from untreated ponds. Unfavorable conditions for the aerobic decomposition of the barley straw may be the reason for the lack of reduction of off-flavor in the catfish. Wills et al. (*16*) suggest that the limited availability of dissolved oxygen in the water and the high oxygen demand in the masses of decomposing barley straw resulted in anaerobic conditions within the large masses of barley straw. This research is still underway; however, aerobic "digesters" are now being used to provide suitable conditions for the aerobic decomposition of barley straw (personal communication with Gene Wills).

## Anticyanobacterial Activity of Lysine

Kayo and Sano (*17*) determined that L-lysine, a component of yeast extract, was toxic towards the cyanobacteria *Microcystis viridis*, *Microcystis aeruginosa*,

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**Table I. Fatty Acids Screened to Determine Selective Toxicity towards *Oscillatoria peronnata***

| | *Test Organism* | | | |
|---|---|---|---|---|
| | *Oscillatoria perornata* | | *Selenastrum capricornutum* | |
| *Compound* | *LOEC (μM)* | *LCIC (μM)* | *LOEC (μM)* | *LCIC (μM)* |
| Arachidonic acid | 10 | 100 | >100 | >100 |
| Docosahexaenoic acid | 0.01 | 100 | 0.01 | 100 |
| Elaidic acid | >100 | >100 | >100 | >100 |
| Erucic acid | 1.0 | 1.0 | >100 | >100 |
| Lauric acid | 10 | >100 | >100 | >100 |
| Linoleic acid | 10 | 100 | 100 | >100 |
| Linoleic acid, sodium salt | 100 | 100 | >100 | >100 |
| Linolenic acid | 0.01 | 1.0 | 100 | >100 |
| 1-Monolauroyl-*rac*-glycerol | 100 | 100 | >100 | >100 |
| Myristic acid | 10 | >100 | >100 | >100 |
| Nervonic acid | 0.01 | 100 | >100 | >100 |
| Nonanoic Acid | 10 | 10 | 10 | 10 |
| Octanoic acid | 10 | 100 | 100 | >100 |
| Octanoic acid, sodium salt | 0.01 | 1000 | >100 | >100 |
| Oleic acid | 0.1 | 100 | 100 | >100 |
| Oleic acid, sodium salt | 100 | 100 | >100 | >100 |
| Palmitic acid | >100 | >100 | >100 | >100 |
| Palmitic acid, sodium salt | >100 | >100 | >100 | >100 |
| Palmitoleic acid | 10 | 10 | >100 | >100 |
| Petroselenic acid | 10 | >100 | >100 | >100 |
| Stearic acid | >100 | >100 | >100 | >100 |

NOTE: LOEC = Lowest-observed-effect concentration; LCIC = Lowest-complete-inhibition concentration.

and *Microcystis wesenbergii* at concentrations as low as 1 ppm (6.85 μM). However, 1 ppm of L-lysine was not toxic to another cyanobacterium *Anabaena flos-aquae* and a green alga *Chlorella vulgaris*. Schrader and Harries (*18*) found that 146 ppm (1000 μM) of L-lysine was toxic towards *O. perornata*, but not at concentrations of 14.6 ppm (100 μM). Similarly, Zimba et al. (*19*) found that L-lysine at or below 100 ppm (685 μM) did not alter chlorophyll *a* levels in *Planktothrix perornata* [another taxonomic name for *O. perornata* as reassigned by Anagnostidis and Komárek (*20*)], *M. aeruginosa*, and the MIB-producing cyanobacterium *Pseudanabaena articulata*. The green alga *Scenedesmus dimorphus* and the brown alga *Cylcotella meneghiniana* were also tested and found to be less sensitive to L-lysine additions to the cultures compared to the cyanobacteria tested. D-Lysine was found to have no effect on any of the taxa tested. The authors propose that exogenous L-lysine may inhibit enzymatic processes due to the isomeric specificity of the inhibition and a decrease of the inhibitory effects of L-lysine as inoculum (*M. aeruginosa* culture) concentration was increased. The high concentrations required to kill *O. perornata* in the laboratory suggest that L-lysine is not very suitable as an algicide for catfish aquaculture.

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## Evaluation of Ferulic acid as a Selective Algicide

As mentioned earlier, Schrader et al. (*15*) screened a variety of natural compounds including several phenolic compounds that are decomposition products from straw cell walls. Sinapic acid and *trans*-ferulic acid (Figure 2A) were found to be selectively toxic towards *O. perornata*. Ferulic acid was toxic (adversely affected growth) toward *O. perornata* at 1 μM while concentrations as high as 1000 μM were required before toxicity towards the green alga *Selenastrum capricornutum* was observed. Of the three types of ferulic acid tested (*cis*-ferulic acid, *trans*-ferulic acid, and methyl ester of *trans*-ferulic acid) to determine their relative toxicity towards *O. perornata*, *trans*-ferulic acid was the most toxic (*21*). Efficacy testing of *trans*-ferulic acid was performed to determine its usefulness as a selective algicide in catfish production ponds (*22*). Results found that applications of ferulic acid to catfish ponds at approximately 5 μM did not affect the abundance of *O. perornata* or MIB levels in pond water. The lack of activity of ferulic acid in the catfish ponds was attributed to its rapid dissipation from the pond waters (its half-life in the pond water column was approximately 2 hours). Ferulic acid may have undergone microbial degradation and/or chemical transformation. The rapid dissipation of some natural compounds in catfish ponds is one potential drawback to their effectiveness as selective algicides. A sufficient amount of persistence of the natural compound

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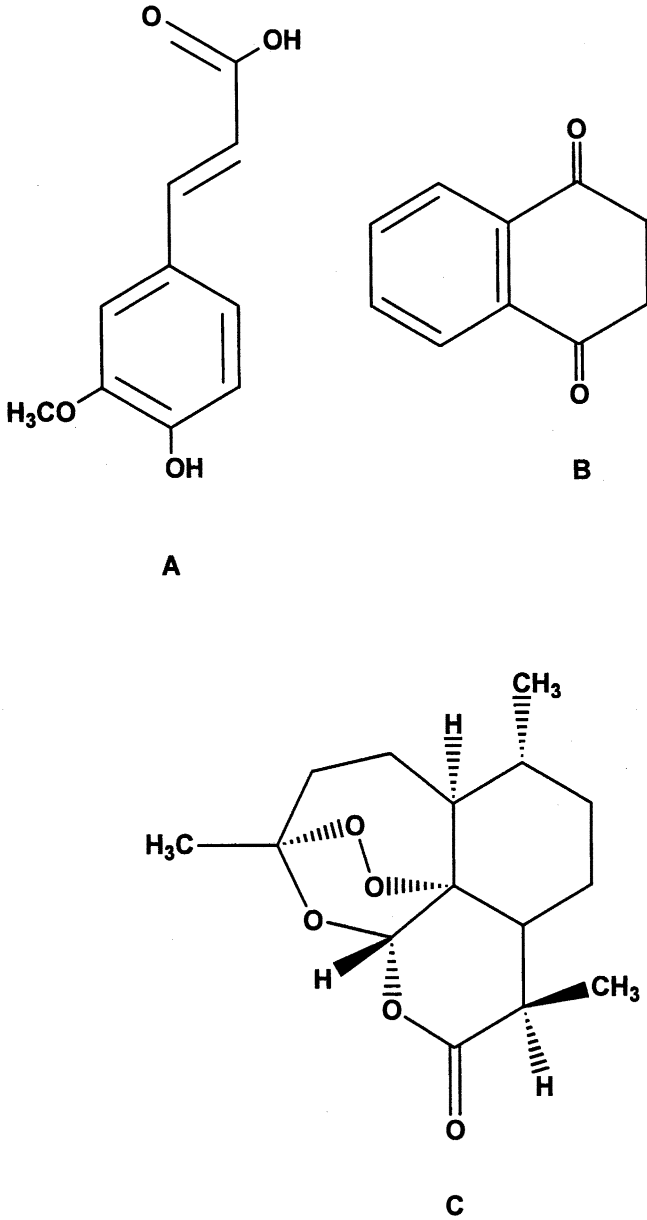
O
OH
$H_3CO$
OH
A
O
O
B
$CH_3$
H
$H_3C$
O
O
O
H
O
$CH_3$
H
O
C

*Figure 2. Chemical structures of trans-ferulic acid (A), anthraquinone (B), and artemisinin (C).*

in the pond water may be required to permit uptake by the undesirable cyanobacteria.

## Evaluation of Quinones as Selective Algicides

Fitzgerald et al. (*23*) were the first to report of the algicidal activity of certain quinones towards bloom-producing species of cyanobacteria including *M. aeruginosa*, *Microcystis incerta*, *Anabaena circinalis*, *Gloeotrichia echinulata*, and *Aphanizomenon flos-aquae*. Specifically, they found that 2,3-dichloronapthoquinone and phenanthraquinone were toxic towards *M. aeruginosa*, a toxin producer commonly found in freshwater ecosystems, at concentrations as low as 2 μg/L (8.6 nM) and 80 μg/L (0.4 μM), respectively. More recently, several quinones have been found to be selectively toxic towards *O. perornata* (*15, 18*). Anthraquinone (Figure 2B), found in some plant tannin extracts (*24*), was among the most selectively toxic compounds screened with complete growth inhibition of *O. perornata* at 0.1 μM or approximately 21 μg/L (*15*). Concentrations as high as 100 μM of anthraquinone were not toxic towards *S. capricornutum*. The mechanism of toxicity of anthraquinone towards *O. perornata* has also been investigated by observing structural changes in *O. perornata* and monitoring chlorophyll fluorescence as an indicator of photosynthetic efficiency (*25*). The results found that anthraquinone inhibits photosynthetic electron transport, possibly at photosystem II, and subsequently inhibits growth. Fitzgerald et al. (*23*) attribute the toxicity of quinone-type compounds to their redox character, but they also hypothesize that some additional toxic property must account for the large degree of selective toxicity towards cyanobacteria of some of the quinones tested. Their studies also found that not all compounds with a quinone structure and/or redox property are highly toxic towards cyanobacteria. Based upon the results of their screening studies, Fitzgerald et al. (*23*) state that the relationship between the toxicity and chemical structure of quinone compounds can be summarized in two principles. First, any compound with two H atoms that can be oxidized from hydroxyl or amino groups that are *ortho* or *para* to each other, adjacent, or that have some similar relationship will yield a stable oxidant that is toxic. Secondly, the structure of the rings and the additions to the rings will increase or decrease the toxicity due to the effects on the redox character. Toxicity will be increased by *ortho* or *para* directing groups in the aromatic ring that will weaken the bond between the H atom and the O or N atom. Conversely, toxicity is decreased by *meta* directing groups that will weaken hydrogen bonding to the O or N atom.

Results from the screening of additional quinones by the method of Schrader et al. (*7*) to determine toxicity towards *O. perornata* are in Table II (K.K. Schrader, previously unpublished observations). Many of the quinones listed in

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Table II are not natural compounds and are not very toxic towards *O. perornata* compared to anthraquinone.

## Efficacy Testing of Anthraquinone

Because of such a high degree of selective toxicity towards *O. perornata* in laboratory studies, anthraquinone was designated as a lead compound for efficacy testing in catfish ponds. Limnocorrals and the method of Schrader et al. (*26*) were used to conduct pond studies to determine if anthraquinone is suitable for use as a selective algicide in catfish ponds (unpublished observations). Because anthraquinone is not soluble in water, different formulations of anthraquinone were prepared in order to maintain it in the water column. The formulations of anthraquinone that were made and tested include the following: 1) solubilization of anthraquinone in ethanol; 2) anthraquinone, canola oil, and Tween® 80 emulsion; and 3) anthraquinone attachment to hydroxylpropylmethylcellulose (HPMC), a neutral binding agent, followed by solubilization of the complex in ethanol. The final concentration of anthraquinone for each type of formulation tested within the limnocorrals was 5 µM, a much higher concentration than the 0.1 µM anthraquinone that killed *O. perornata* in microplate bioassays (*15*). Results found that none of the anthraquinone formulations tested were effective in reducing the abundance of *O. perornata* in pond water within treatment limnocorrals. For several days after the application of each test formulation, anthraquinone levels remained above 0.1 µM in the water column. It is possible that the formulations tested did not provide anthraquinone in a readily available state for uptake by cells of *O. perornata*. Other types of anthraquinone formulations have not been tested in catfish ponds yet. Consequently, water-solubility limitations of certain natural compounds found to be very effective in killing *O. perornata* in laboratory bioassays presents a dilemma for their effectiveness as selective algicides in catfish ponds.

## Anticyanobacterial Activity of Artemisinin

The antimalarial compound artemisinin (Figure 2C), a sesquiterpene lactone, was found to be selectively toxic towards *O. perornata* at 1 µM in microplate bioassays (*15*). The plant *Artemisia annua*, commonly referred to as annual wormwood, produces artemisinin (*27*). Levels of artemisinin are approximately 4 to 11 times higher in the inflorescences of *A. annua* than in the leaves or stems (*28*). Due to its high cost, purified artemisinin is not economically practical for use in catfish ponds as an algicide. For a more

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**Table II. Quinones Screened to Determine Selective Toxicity towards *Oscillatoria perornata***

| | *Test Organism* | | | |
|---|---|---|---|---|
| | *Oscillatoria perornata* | | *Selenastrum capricornutum* | |
| *Compound* | *LOEC ($\mu$M)* | *LCIC ($\mu$M)* | *LOEC ($\mu$M)* | *LCIC ($\mu$M)* |
| 1-Aminoanthraquinone | 100 | 100 | >100 | >100 |
| 2-Aminoanthraquinone | 100 | >100 | >100 | >100 |
| Anthraquinone-1,5-disulfonic acid | 1000 | >1000 | >1000 | >1000 |
| Anthraquinone-2-carboxylic acid | 100 | 100 | 100 | 1000 |
| 9,10-Anthraquione-2-sulphonic acid | 100 | 1000 | >1000 | >1000 |
| Anthrarufin | 1 | 10 | >100 | >100 |
| 1,2,4-Benzenetriol | 100 | 100 | 0.1 | 1000 |
| 1,4-Benzoquinone | 100 | 100 | >100 | >100 |
| 2-Bromo-3-hydroxy-1,4-naphthoquinone | >100 | >100 | 100 | >100 |
| 3-Bromo-5-*tert*-butyl-2-chloro-1,4-benzoquinone | 10 | 10 | 100 | 1000 |
| 2-*tert*-Butyl-1,4-benzoquinone | 10 | 100 | 1000 | >1000 |
| 2-*tert*-Butyl-3-chloro-5-methyl-1,4-benzoquinone | 10 | 10 | 1000 | 1000 |
| 2-*tert*-Butyl-5-methyl-1,4-benzoquinone | 1 | 10 | 1000 | 1000 |
| 3-*t*-Butyl-5-methoxy-*o*-benzoquinone | >100 | >100 | >100 | >100 |
| 4-*t*-Butyl-5-methoxy-*o*-benzoquinone | >100 | >100 | >100 | >100 |
| 2-Chloro-3-hydroxy-1,4-naphthoquinone | 100 | 100 | 100 | 1000 |
| Chlorohydroquinone | 10 | 10 | >100 | >100 |

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| | | | | |
|---|---|---|---|---|
| 2,5-Dichloro-1,4-benzoquinone | 100 | 100 | 100 | 1000 |
| 1,4-Dihydroxyanthraquinone | >100 | >100 | >100 | >100 |
| 1,8-Dihydroxyanthraquinone | 100 | 100 | 100 | >100 |
| 5,8-Dihydroxy-1,4-naphthoquinone | 100 | 100 | >100 | >100 |
| 2-Hydroxy-1,4-naphthoquinone | 1000 | 1000 | 100 | 1000 |
| 2-Hydroxy-3-(5-methylhexyl)-1,4-naphthoquinone | 10 | 100 | 100 | 100 |
| 2-Hydroxy-3-(3-methylbutyl)-1,4-naphthoquinone | 1000 | 1000 | 100 | 100 |
| 2-Hydroxy-3-(2-phenylbutyl)-1,4-naphthoquinone | 100 | 100 | 100 | 100 |
| 2-Hydroxy-3-(5-methylhexyl)-1,4-naphthoquinone | 100 | 100 | 100 | 100 |
| 2-Hydroxy-3-isobutyl-1,4-naphthoquinone | 100 | 1000 | 100 | 100 |
| 2-Hydroxymethylanthraquinone | 10 | 10 | 100 | 100 |
| 2-Hydroxy-3-methyl-1,4-naphthoquinone | 100 | 100 | 100 | 100 |
| Lapachol | >100 | >100 | >100 | >100 |
| 1-(Methylamino)-anthraquinone | 10 | 100 | >100 | >100 |
| 2-Methyl-1,4-naphthoquinone | 10 | 10 | 100 | 100 |
| 1,2-Naphthoquinone | 100 | 100 | >100 | >100 |
| Plumbagin | 10 | 10 | 100 | 100 |
| 2,3,5,6-Tetrachloro-1,4-benzoquinone | 10 | 10 | 100 | 100 |
| *p*-Tolyol-*p*-benzoquinone | 1 | 10 | 10 | 100 |

NOTE: LOEC = Lowest-observed-effect concentration; LCIC = Lowest-complete-inhibition concentration.

economical approach, the use of *A. annua* inflorescences to kill *O. perornata* was studied. Although artemisinin is only sparingly soluble in water, inflorescences (44 μg dry weight/mL of culture) from *A. annua* placed in 100-mL batch cultures of *O. perornata* resulted in the death of the cyanobacterial cells after two days (K.K. Schrader, unpublished observations). Artemisinin and/or some other natural compound(s) could have been responsible for this toxicity. Previous research has determined the artemisinin content of dried *A. annua* inflorescences (*28*). The amount of inflorescences used in the study was such that artemisinin levels were expected to be above 1 μM, a concentration completely toxic to *O. perornata* in laboratory bioassays (*15*). A pond study was conducted using the method of Schrader et al. (*26*) to determine if inflorescences from *A. annua* might be useful as a selective algicide. The inflorescences were placed inside miracloth (Calbiochem, La Jolla, CA) bags (80 g dry weight inflorescences per bag), and three bags were placed on the water surface within each treatment limnocorral (three treatment replicates and three controls). The amount of inflorescences used for each treatment was equivalent to the amount used in the laboratory study on a dry weight per volume basis. The abundance of *O. perornata* and MIB levels within treatment limnocorrals were not reduced by the addition of *A. annua* inflorescences to the pond water. Therefore, *A. annua* inflorescences at the application rate used in this study are not useful to help control musty off-flavor problems in farm-raised catfish.

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## Screening of Plant Extracts for Algicidal Activity

Several plant extracts have been screened for toxicity towards *O. perornata* to determine if they contain natural compounds useful as selective algicides. The ether and hexane extracts of tarbush (*Flourensia cernua*) showed complete inhibition of *O. perornata* in laboratory bioassays (*29*). The essential oil from beautyberry or French mulberry (*Callicarpa americana*) was found to be selectively toxic towards *O. perornata* (*30*). The active compounds in these extracts have not yet been determined.

## Conclusions

A small percentage of the vast numbers of natural compounds have been studied so far to determine their potential for use as selective algicides in catfish aquaculture. Previous research indicates that there is great potential for discovering natural, selective algicides. The selection and development of promising natural compounds for use as commercial algicides in catfish aquaculture must consider several issues. Efficacy studies must show that the algicidal compound is reliable and relatively inexpensive to use and apply in catfish ponds. The algicidal compound must be effective and selective in controlling the undesirable cyanobacteria species at a fairly low concentration (µg/L range), and the environmental persistence of the compound must not be too lengthy. In addition, the algicidal compound and the "breakdown" products of the compound must not be toxic towards non-target organisms (e.g., catfish). The accumulation of any residues from the algicidal compound in farm-raised catfish must also be known.

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## Chapter 15

# Applications of an Instrumental Method for the Analysis of Off-Flavors in Fresh Water Aquaculture

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Off-flavors in aquaculture are problematic because of economic loss at the production level and potential for consumer avoidance of an unsatisfactory product reducing sales. The most common off-flavors in fresh water products are muddy, musty aromas resulting from the uptake of 2-methyl-isoborneol (2-MIB) and geosmin (GSM) from the water column. These compounds are metabolic products produced by cyanobacteria. The catfish industry in particular is affected by off-flavor problems. Shrimp farmers tend to see these problems only during periods when associated with fresh water incursion. An instrumental method for analyzing GSM and 2-MIB has been developed. Detection levels from tissue samples are less than 50 ng/kg for 2-MIB and GSM, and approach 1 ng/kg in water. These levels are sufficient for determining consumer acceptability. The technique was applied to monitor seasonal changes in concentration levels of 2-MIB in catfish ponds, the effects of storing samples, and the uptake and purging of these off-flavors between water and fish.

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## Introduction

Over 40 species of algae are known to produce off-flavors compounds (*1, 2*). Algae, particularly cyanobacteria, are known to produce these compounds within aquatic systems (*3, 4*). Particularly problematic is the production of geosmin (GSM) and 2-methylisoborneol (2-MIB). These compounds can be produced by fungi or by cyanobacteria and are ubiquitously distributed in nature (5, *6*). These semi-volatile compounds have a muddy, musty odor and are discernible by the human nose when present at minimal detectable concentrations of 0.004 - 0.020 µg/L in water (7, *8*). Although posing no known health hazard (*9*), municipal water systems are plagued with consumer complaints when these compounds exceed the threshold of human perception. Additionally, both compounds are rapidly absorbed from water into the adipose tissue of fish and other aquatic organisms and, when present in tissue at concentrations greater than ca. 0.6 µg/kg, render fish unfit for retail consumption (7). At present, economical means are not available to prevent production and accumulation of these compounds by aquatic organisms (10).

Traditional analytical methods for monitoring 2-MIB and GSM concentrations include closed-loop stripping (11), liquid-liquid extraction (12), steam distillation (*13)* and purge and trap (*14*). These techniques are effective, but are expensive, time consuming and labor intensive. Three alternative techniques have recently been introduced but have not gained broad acceptance. Membrane-based extraction (*15*) can detect analytes in the parts per trillion concentration ranges while solid phase extraction (*16*) is rapid, inexpensive and can detect concentrations at the parts per billion levels. A third alternative is solid phase micro-extraction (SPME), a method pioneered by Pawliszyn and colleagues (*17*).

SPME is a simple and inexpensive method for the analysis of volatile and semi-volatile compounds occurring in a wide variety of food, water and environmental matrices (*18*). A fused silica fiber is coated with a suitable adsorbent phase and bound to the tip of a syringe plunger. The plunger is retracted into the needle, thereby protecting the delicate fiber. The needle is used to pierce the septum of a sealed vial containing the sample and the SPME fiber is then extended (see Figure 1). Volatile and semi-volatile compounds are allowed to equilibrate between the sample, the headspace and the fiber. Analyte molecules are absorbed onto the coating. After equilibration, the fiber is retracted into the needle and inserted into the heated injection port of a gas chromatograph. The analytes are thermally desorbed and transferred onto the head of a capillary column in a gas chromatograph for subsequent separation and detection.

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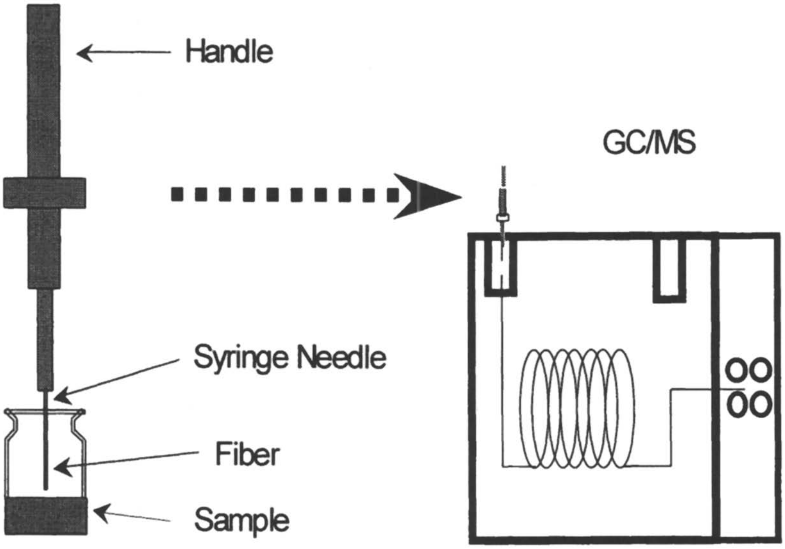

*Figure 1. Volatile compounds are adsorbed onto a fiber using solid phase microextraction. The volatile compounds are subsequently desorbed into the injection port of a GC/MS system.*

In addition to headspace analyses the fiber can be directly immersed into a liquid sample. However, formation of charge zones around the fiber prevents equilibration during liquid immersion and limit the amount of analyte adsorbed by the fiber (*19*). Additionally, adsorption of non-volatile compounds on the fiber can result in their thermal degradation during the thermal desorption and can complicate the resulting analysis. Quantitative analysis using SPME is problematic as two sets of equilbria must be obtained for a single analyte and multiple compounds result in competition for fiber sites (*20, 21*).

Employing the SPME headspace technology, we have developed a method for detecting and quantifying trace levels of 2-MIB and GSM in water and in fish tissue. Use of SPME extraction procedures increased laboratory throughput by 4-5 fold over the purge and trap method previously used (*22*). One of our goals in the development of rapid analytical methodologies was to increase research capabilities; we also hoped to identify relationships between off-flavor in pond water and fish. Our intent was to develop simple regression relationships for prediction of off-flavors, thereby offering an alternative to conventional flavor checking within fish processing facilities.

## Methods

### Solid Phase Micro-Extraction of Water

Water samples were collected from three separate locations within a pond at six inches below the surface and pooled. Aliquots consisting of 600 µL of unfiltered water were placed into a 2 mL glass vial. Sodium chloride (NaCl) was added in an amount sufficient to saturate the solution (~0.3g). The sample was heated to 65°C and exposed to the SPME fiber for a 12-minute adsorption period while being vigorously agitated. The auto sampler was equipped with a 1 cm long, divinylbenzene/carboxen/polydimethylsiloxane SPME fiber (Supelco, Inc., Bellefonte, PA). The fiber was withdrawn from the sample and desorbed at 270°C for 5 minutes in the injection port of an HP6890 gas chromatograph, quipped with a 5973 mass selective detector (Agilent Technologies, Palo Alto, CA). The injection port was operated in pulsed splitless mode and fitted with a 0.7 mm ID injection liner. The head pressure was set to 25 psi of helium for the first minute, and then to a constant velocity of 40 cm/s for the remainder of the GC run. A 30 m, 0.25 mm I.D., with a 1.0 µm film consisting of 5% diphenyl, 95% polydimethylsiloxane was employed as the stationary phase. Two different GC temperature programs were employed, one for qualitative analysis and the second for quantitative analysis. For qualitative analysis the oven was held at an initial temperature of 40°C for 3 minutes then increased at 5°C/min to 200°C then at 50°C/min to 250° and held for total run time of 40 minutes. For quantitative analysis using selected ion monitoring (SIM), the oven was initially held at 80°C for one minute then increased 20°C/min to 100°C, 7.5°C/min to 152°C, 65°C/min to 250°C, where the oven was held to give a total run time of 12.75 minutes. Cool down for the GC oven took approximately 4 minutes.

### GC/MS Analysis

The quadrupole mass spectrometer was operated in electron ionization mode and was initially scanned from *m/z* 50 to *m/z* 350 for qualitative analysis. SIM was employed for quantitation for the target and qualifier ions for 2-MIB, GSM, and for the internal standards, borneol, and decahydro-1-naphthol. Quantitation was based on the base peak of each analyte and standard, 2-MIB: *m/z* 95, GSM *m/z* 112, borneol: *m/z* 95 decahydronaphthol: *m/z* 136. Qualifying ions were employed for quality control to assure a consistent ratio relative to the target ion. The molecular ions were used as the qualifying ions, 2-MIB: *m/z* 168, GSM *m/z* 182, borneol: *m/z* 154 decahydronaphthol: *m/z* 154. If the ratio fell outside of acceptable limits (5-10%), the presence of a co-eluting compound was suspected which could have interfered with the quantitation. Following the first GC/MS

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run, subsequent samples were prepared in advance resulting in sample analysis time of 18 minutes. To eliminate carryover, after washing, glassware was rinsed with a 1M HCl solution, followed by a water rinse and baked at 200°C.

Geosmin, (9a, 10a-decalol; CAS#: 19700-21-1) was obtained from Givaudan Corporation (Clifton, NJ). 2-Methylisoborneol, ([1R-*exo*]-1,2,7,7-tetramethyl - [2,2,1]-bicyclo-heptan-2-ol; CAS#: 2371-42-8), borneol ([1R]-endo-1,7,7-Trimethyl bicyclo[2.2.1] heptan-2-ol; CAS#: 464-43-7) and decahydro-1-naphthol (*cis*-decahydro-1-naphthol; CAS#: 36159-47-4) were obtained from Sigma-Aldrich (St. Louis, MO). For pure compounds, initial stock solutions were prepared in ethanol to ≅ 1 part per thousand. Subsequent dilutions were made using Milli-Q water (Millipore, Bedford, MA). For water analysis, standards were prepared over the 0.01 to 10 ppb range. For analysis of muscle tissue, standard dilutions were made at 0.1, 0.5, 1.0 1.5, and 3.0 ppb.

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### Solid Phase Micro-Extraction of Fish

Off-flavor fish were obtained from a commercial processor and determination of the muddy, musty, off-flavor was made by a professional flavor checker. Samples consisted of twenty grams of a single catfish fillet that was finely chopped and placed in a specialized glass container (*22*). Each sample was spiked with 5 µL of a 10 mg/kg aqueous solution of borneol as an internal standard. The sample was then heated for three minutes in a 370W microwave set at 50% power, while purging with 80 mL/min of $N_2$. The effluent was transferred via glass tubing to a receiving vessel (25 ml graduated cylinder) in a chilled water bath held at 0°C. The condensed water was brought up to a total volume of 10 mL using deionized water used to rinse the transfer line. The sample was then divided into 5 mL aliquots and each placed into a 10 mL vial. Three grams of NaCl were added and the vial spiked with 5 µL of a 10 ppm aqueous solution of the internal standard, decahydro-1-naphthol (50 ng). The vial was sealed with a crimp cap fitted with a Viton septum and placed in a CTC SPME autosampler (Leap Technologies, Carrboro, NC). Samples were maintained at room temperature until analyzed using the same procedure outlined above.

## Discussion

Whereas the odor threshold for 2-MIB and GSM in water approaches 1 ng/kg, the acceptable taste threshold for 2-MIB and GSM in catfish is considered to be ~ 700 ng/kg for the average consumer (*23*). At this concentration, 50% of people can detect the presence of 2-MIB. However, professional flavor checkers are typically more sensitive to these compounds and can detect their presence at lower concentration levels. This superior ability to detect these off-flavors could be innate or in some cases refined through training (*24*). Although the threshold value would be expected to vary from one person to the next, the taste threshold for a "theoretical average flavor checker" has been determined to be between 0.1 and 200 ng/kg (*25*). Since the lower limit of detection is also ~ 1 ng/kg, even if these off-flavor compounds can be detected in the water, they may not be present in sufficient concentrations in the fish to cause problems.

GSM and 2-MIB are hydrophobic and solubility is less than 1 ppm in water. As a result, rapid diffusion occurs from water to air. Additionally these compounds are lipophilic and do not readily diffuse from fish tissue into the atmosphere. The chemical properties of these compounds suggest that it is easier to purge these compounds from water than fish tissue. Our experience in the laboratory confirms the ease of off-flavor measurement from water by SPME, whereas direct headspace analysis of fish tissue yields poor results even for extremely off-flavor fish. During consumption, 2-MIB and/or GSM is released from the tissue during mastication. The compounds pass through the retronasal cavity to the olfactory bulb, where they are sensed. In the laboratory, their release can be accomplished by microwave distillation; this technique effectively removes the analytes into the aqueous distillate. Processing time for water is less than 20 minutes and is automated, whereas fish analyses are much more labor intensive and require specialized glassware and additional time (*22,26*). We have used these techniques in several studies examining off-flavor (*1, 4, 26, 27*).

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## Storage of Water Samples

As noted earlier, 2-MIB and GSM are hydrophobic compounds, and are readily volatilized from water. They are immiscible in water, necessitating that standard solutions be prepared in organic solvent such as methanol or ethanol. Over time, 2-MIB and GSM will be expelled from the water column. The volatilization rate is dependent upon a number of variables including temperature, salinity, depth and mixing conditions. Pond water samples, stored in vials with Teflon lined, screw-top caps, and known to contain 2-MIB when sealed, had undetectable levels after several months' storage. Loss could result from leaking of the screw cap vials or catabolic metabolism. To ensure the consistent shelf life of stored samples, an experiment measured the concentration of a series of samples over an extended time period. NaCl was added to form a saturated solution, which should inhibit any catabolic metabolism. Crimp-top vials were used in place of screw cap vials to ensure that volatile compounds did not escape from the vials. Table I shows the measured concentration of 5 g/kg solutions of standards that were made up and sampled over a five-week period.

Table I. Recovery of 5 ppb 2-MIB.

| | *Day 1* | *Day 7* | *Day 14* | *Day 18* | *Day 38* |
|---|---|---|---|---|---|
| Rep 1 | 4.6 | 4.6 | 5.1 | 4.7 | 5.2 |
| Rep 2 | 4.8 | 4.9 | 4.4 | 5.1 | 4.9 |
| Rep 3 | 4.9 | 5.9 | 5.0 | 5.0 | 5.2 |
| Rep 4 | 5.1 | 5.1 | 4.7 | 5.0 | 4.9 |
| Rep 5 | 5.2 | 5.8 | 5.1 | 5.1 | 5.2 |
| Average | 4.9 | 5.2 | 4.9 | 5.0 | 5.0 |
| STD | 0.2 | 0.5 | 0.3 | 0.2 | 0.2 |

Standard deviations of five repetitions averaged 0.2 g/kg with a maximum value of 0.5 g/kg for day 7. The averaged measurements for each of the time periods are not statistically significant; thus no significant change was observed over this period. The addition of NaCl to samples allows for their extended storage assuming a good seal is obtained on the vial. The integrity of the vial can be checked by ensuring that the crimp cap does not rotate.

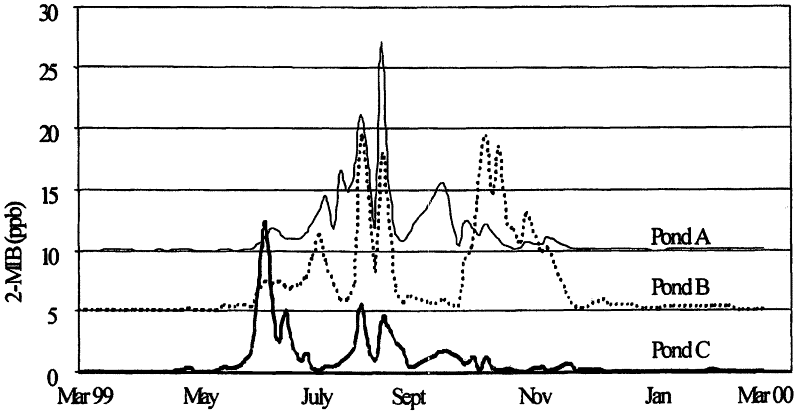

*Figure 2. Concentration of 2-MIB in water samples collected twice per week over a 1-year period from three Louisiana catfish ponds.*

## Seasonality of Off-flavor Occurrence in Water

Strong seasonality of off-flavor occurs in catfish production ponds. The highest occurrence of off-flavor in water occurs during June-October for the

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muddy (also known as blue-green) odor resulting from the presence of 2-MIB and/or GSM. Their presence is highly episodic as a result of the ephemeral succession pattern of the algae (*28*), as well as pressure of grazing, and other environmental conditions. During the winter and spring months little off-flavor results from 2-MIB and/or GSM. However, a woody off-flavor becomes problematic during the winter months. The woody problem is not as offensive and the occurrence is not as pervasive as the muddy odors. The concentration of 2-MIB and GSM was monitored twice a week over a one-year period from March 1999, through February 2000. Figure 2 shows the levels of 2-MIB from three production ponds monitored over a one-year period. Only trace levels of GSM (< 0.050 μg/kg) were detected over that period. Ponds A and B have been offset by 10 and 5 ppb for clarity. Of interest was the rapid change in the 2-MIB concentrations of the catfish ponds. Ponds could become off-flavor in a matter of days and clear up just as quickly. Ponds found with only trace levels of 2-MIB only three days prior can have significant quantities of 2-MIB present to render fish off-flavor during the next sampling period. However, as observed in Pond B from late September until mid-November, the off-flavor problem could linger over a much longer period. These ponds were proximate in location, experienced the same weather, and were under identical stocking and feeding regiments. They clearly demonstrate the highly episodic occurrence of blooms of off-flavor producing cyanobacteria and the resultant high variability of off-flavors in farm raised catfish.

## Storage of Fish Fillets

Fish samples cannot always be immediately analyzed. The question arises as to, what is the best storage method. Since SPME is an equilibration technique in which a change in the composition of the headspace could affect the recovery of the 2-MIB and GSM analytes, the goal is to minimize the changes in the volatile profile. Storing fresh fish on ice or under refrigeration is acceptable for several days at most. In some cases freezing samples can cause changes in the volatile profiles of food samples. To determine if there is any difference in analyzing fresh or frozen samples for 2-MIB, fillets from five different fish were subdivided into three, 20-gram portions. The first portion was analyzed immediately (fresh) and the remaining portions were frozen. Portions from each of the five fish were removed from the freezer and analyzed at 7 and 39 days.

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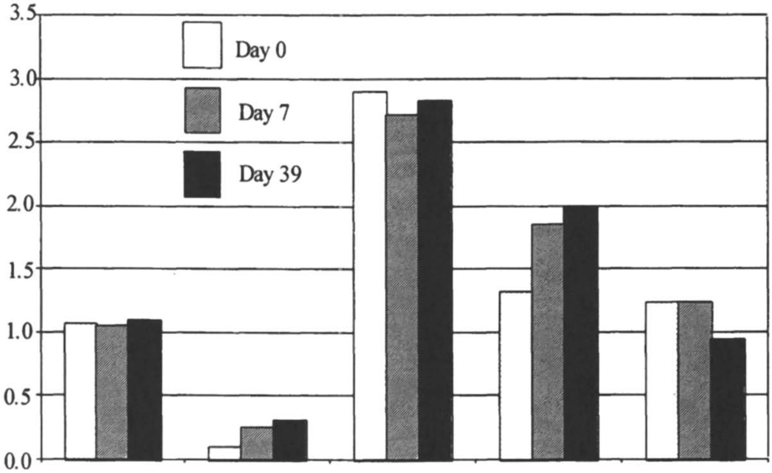

*Figure 3. Comparison of the recovery of 2-MIB from fresh fish (day 0) relative to fish stored under frozen conditions (day 7 and day 39).*

Figure 3 shows the concentrations of 2-MIB determined from fresh and frozen portions of five different fish. Fish one and three show no difference between time points within experimental error (10%), whereas fish two and four show a slight increase in 2-MIB over the storage period. Fish five shows a slight loss relative to the fresh and one week frozen time periods. Overall the data suggest that there is little difference, if any, in the amounts of 2-MIB in fresh and frozen fish. The differences are most likely due to experimental error in the instrumental method (~ 10%). Based on this data, freezing of fish is a good way to preserve samples.

## Concentration in Fish

2-MIB has been reported to be rapidly absorbed by fish (*29*). We examined uptake of 2-MIB and GSM using static raceways (Figure 4). Fish for the experiment were checked for off-flavor and found to contain negligible amounts of 2-MIB or GSM at time zero. Fish were exposed to a concentration of 3.5 ppb of 2-MIB and 3.5 ppb of GSM in each of three raceways. Periodically, 10 fish from each raceway were removed and analyzed for the presence of 2-MIB and GSM. After only 1 hour, concentrations of 2-MIB in the fish readily exceed 1.0 ppb. Maximum levels were achieved after 24 hours of exposure and remained constant over the time period monitored (72 hr). No difference was observed in the concentrations of 2-MIB and GSM if fish at each time period. No evidence of bioaccumulation was found over this time period, as the concentration in fish never exceed the original value in the water. This differs from previous work by Johnsen et al. (*14*) and Martin et al. (*29*).

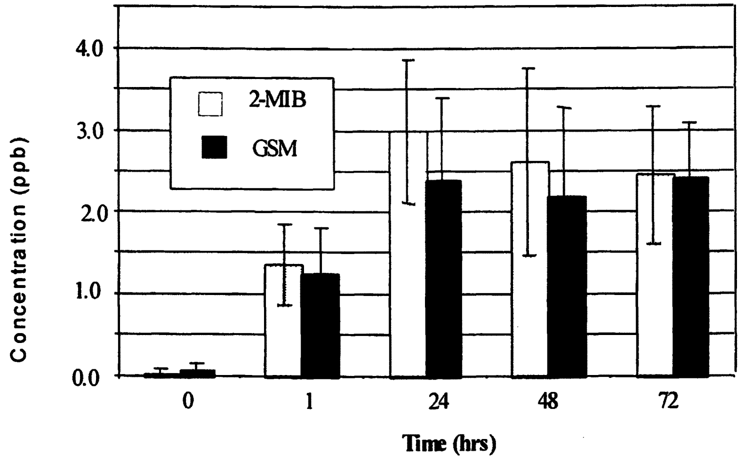

*Figure 4. Uptake of off-flavor compounds by catfish from water spiked with 3.5 µg/kg standards.*

## Purging

For this experiment off-flavor fish were harvested from a commercial pond in late October, 2000. Water and fish samples were collected weekly over a six-week period prior to harvest. Elevated 2-MIB concentrations were confirmed and the fish were removed from the pond and placed in three flow-thru raceways (25 L/minute well-water flow rate) for 96 hours. Ten fish from each raceway were collected at selected time intervals and analyzed for the presence of 2-MIB and GSM. Figure 5 shows the average of the 10 fish samples for each raceway. Within 48 hours purging time, fish contained concentrations of 2-MIB that were below published (acceptable) off-flavor concentrations of 0.7 ppb, however these fish would still be rejected by at least some flavor checkers after 96 hours (>0.10 ppb). Purging of off-flavors occurs more slowly than uptake.

Of peripheral interest during this study were the changing levels of GSM. As noted above, initially no GSM was detected in the fish prior to removal from the pond. However, significant levels of GSM were observed in fish harvested from two of the three raceways. The levels tended to increase with time. It was observed that algae had grown on the side of the two raceways experiencing GSM contamination. Therefore off-flavor problems can occur even during purging if sufficient steps are not taken to keep raceways clear of contaminating bacteria.

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While some processors employ purging to treat off-flavor fish it is not a widely accepted practice. Fish can lose 10-20% of their body weight during the purging period. Additionally, existing processing facilities were not designed to handle off-flavor fish, as sufficient numbers of raceways may not be available for holding fish over extended periods.

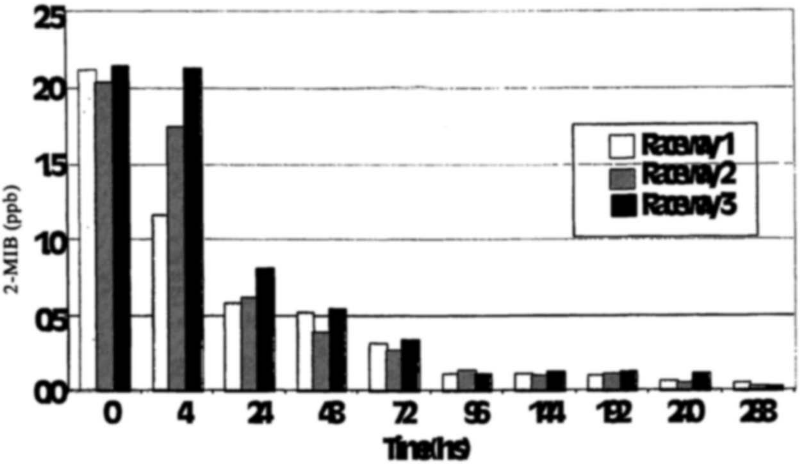

*Figure 5. Average concentration of 2-MIB from 10 fish from each of three raceways purged with fresh water.*

## Water: Fish Off-flavor Relationship

Previous work by van der Ploeg *et al* (*30, 31*) examined off-flavor concentration in water samples on a monthly basis from ponds in Alabama and Mississippi. The highest off-flavor concentrations were observed during summer-fall. Off-flavor in fish was assessed by flavor checking, and pond water was assessed by cell counts of a known off-flavor producing algae (*Planktothrix perornata*). However, no correlations were possible between the subjective flavor scores and objective cell count data.

Several studies have assessed seasonality of off-flavor occurrence in ponds on time scales of days to weeks (*29-30*). A limited number of paired fish and water samples were collected from these studies in Mississippi (n=34) and Louisiana (n=28). In brief, water samples were held in a darkened cooler on ice until processing. Typically processing was completed within 4 hr of collection. Samples of pond water (0.6 or 8 mL) were collected and analyzed in triplicate according to published procedures (*32*). Fish were collected by hook and line, and placed on ice. Fillets were removed from each fish and frozen (-20°C) until analyzed. Fillets were sampled (20.0 g) and analyzed using microwave distillation as previously described (22, 27). Data were analyzed using correlation analyses (*33*).

Correlation analyses between 2-MIB and GSM were calculated for paired water and fish samples (Table II). A significant relationship between 2-MIB concentrations in water and fish was found (p. = 0.012), however the GSM water: fish relationship was not significant (p. = 0.099). It is not surprising that water and fish were not highly correlated. The timing of algal synthesis of off-flavor metabolites, release into the water column, uptake by fish, and depuration may not have been synchronized. Purging of off-flavors from fish is slow relative to uptake. Removal of off-flavor compounds requires greater than four-fold more time than uptake (Figure 3-4). Therefore, algal blooms producing off-flavor may disappear, but the effects of these blooms on fish off-flavor can persist for days to weeks following these events.

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**Table II. Pearson correlation coefficients for 2-MIB and GSM samples.**

| | *2-MIB fish* | *GSM fish* |
|---|---|---|
| 2-MIB water | 0.4251<br>p. = 0.012 | -0.09595<br>p. = 0.6275 |
| GSM water | 0.2291<br>p. = 0.908 | 0.3182<br>p. = 0.0990 |

*A total of 34 paired observations were used for 2-MIB, 28 paired samples for GSM were included in the analyses.*

## Summary

SPME analysis of the off-flavor metabolites, 2-MIB and GSM, provide, a more rapid method of assessment than previously available instrumental methods. Flavor checkers, although currently faster, are not as sensitive and lack the precision inherently available in an instrumental method. The method modified for analyses of fish samples increased laboratory throughput 4-5 fold over previous instrumental methods. The SPME GC/MS instrumental method can detect concentrations in water at the 1 ng/kg level.

The concentration of off-flavor compounds in catfish ponds is highest during June-October. Fish can rapidly absorb these compounds and become off-flavor even over a 1-hour exposure period. Depuration from fish requires time periods of 3-10 fold longer relative to uptake. This difference may explain why poor off-flavor correlations were observed between water and fish. For practical purposes, it is safe to assume that if 2-MIB and GSM can be detected in the water, they are in the fish at comparable or greater levels in the fish. The converse is not necessarily true.

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## Chapter 16

# Major Causes of Shrimp Spoilage and Methods for Assessment

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Although orgaonoleptic evaluation remains the method utilized most to determine freshness, several techniques show promise in spoilage detection including impediometric measurement and electronic sensor analysis. Shrimp spoils (develops foul odor, becomes discolored and loses texture) primarily due to the actions of microorganisms, proteolytic enzymes, and unsaturated fatty acid autooxidation. Storage temperature affects the type of microflora that predominate on shrimp, and subsequent odors produced. The most offensive spoilage odors are due to combinations of volatile amines, sulfides, and short chain fatty acids. Inhibition of proteolytic enzymes may promote increased shrimp shelf-life.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

# Trends in shrimp consumption and production

Whether boiled with Cajun spices, breaded with cornmeal and deep-fried, or sautéed with butter and garlic, foods prepared with shrimp are extremely popular in the U.S. as they are in the rest of the world. Per capita consumption of shrimp in the U.S. was estimated at 2.8 pounds per person, per year in 1998 (1). Compared to 1979 (1.3 lbs per person), consumption has more than doubled. U.S. demand reached a record high 404,154 metric tons in 1999, surpassing the previous years figure (393,809 metric tons) and maintaining an upward trend consistent since 1995. The U.S. was the world's largest shrimp importer in 1999 (2).

The increased popularity of shrimp is proportional to its increased availability. The quantity of shrimp harvested from the world's oceans and aquaculture facilities increased from 2.4 million metric tons in 1987 to 3.5 million tons in 1997. China leads in shrimp production and Thailand leads in shrimp aquaculture production. The U.S. ranks seventh in total shrimp production behind China, Indonesia, Thailand, India, Vietnam, and Ecuador.

# Spoilage

As with other crustaceans (lobster, crab, crayfish), acceptable post harvest quality of shrimp is difficult to sustain. Ideally shrimp should be prepared and consumed within a short period of time after harvest, otherwise they must be kept cold or rapid deterioration can occur due to any or all of the following: microorganism growth, endogenous enzymatic activity or chemical degradation (auto-oxidation) associated with unsaturated fatty acid content. Shrimp may be considered spoiled when it does not smell fresh, loses texture, or becomes discolored. Of these three sensory attributes, spoilage odors are arguably the most offensive and they serve an important purpose in that they provide a clear warning that the product is deteriorated.

## Spoilage due to microorganisms

Microorganism growth in shrimp may cause spoilage due to metabolic by-product generation. Microorganism by-products include volatile and non-volatile compounds, and possibly toxins. In addition microbes can cause shrimp tissue breakdown resulting in loss of texture and discoloration. Structural degradation of shrimp tissue is due in part to the actions of microbial proteolytic enzymes. Detection of microbial by-products (such as indole, Figure 1) is

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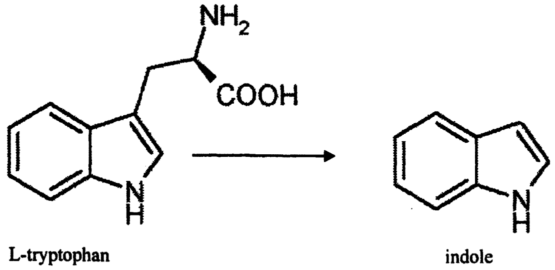

*Figure 1 Microorganism conversion of L-tryptophan to form indole*

commonly utilized as a means for determining shrimp freshness or quality. This technique will be discussed in greater detail in the "Freshness indicators" section.

## Temperature effect

Studies have reported the dominant microorganisms associated with shrimp spoilage are primarily psychrotrophic (bacteria that are capable of growing at 7°C or less and produce a variety of enzymes that cause chemical deterioration) *Pseudomonas* and *Moraxella/Acinetobacter* species (3, 4). However, the aquatic origin (location) of shrimp, as well as water temperature, are important parameters in determining the species of microorganism that predominates. Microorganisms isolated from Black Tiger (*Penaeus monodon*) shrimp harvested from warm Asian waters (Black Tiger accounts for approximately 56% of all shrimp consumed) typically are Gram-positive, and include *Micrococcus*, coryneforms, and *Bacillus*. On the other hand, shrimp harvested from cold waters, including Pink shrimp, *Pandalus borealis*, (80% are harvested from Alaskan waters) typically contain Gram-negative microbes and include the psychrotrophs: *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Flavobacterium*, and *Vibrio* (3, 4, 5). Research also indicates the dominant microflora on shrimp change with changing storage temperatures. Under refrigerated storage (6° C), *Pseudomonas* is the dominant genus. As the temperature increases, *Moraxella* sp. are more abundant, and when temperatures

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are near 22° C (room temperature) *Proteus* species are dominant (4, 6). This trend may be important in explaining spoilage patterns associated with shrimp.

Due to chill adaption, indigenous flora found on cold-water fish species are not inhibited as effectively by refrigeration as are the normal flora of fish harvested from warm tropical waters. When handled properly, tropical fish species are generally less prone to rapid spoilage and exhibit a longer refrigerated shelf life than cold-water species (7, 8).

Storage temperature determines the type of microflora that predominate and resulting volatile compounds that are produced as metabolic byproducts during spoilage. We report here the results of a study conducted in our laboratory where homogenized and heat sterilized freshly harvested Pacific white shrimp *(Penaeus vannamei)* were separately inoculated with different bacteria that had previously been isolated from shrimp maintained at 30° C for 24 hrs. Each sample was divided to form 2 additional samples, one was stored at 6° C for 24hrs and the other at 30° C for 7 days. Sensory analyses and analytical analyses of samples indicated those inoculated with *Chryseomonas luteola* and *Serratia marcescens* and maintained at 30° C for 24 hrs possessed the strongest and most unpleasant aroma. The aroma of inoculated samples maintained at 6° C for 7 days were not significantly different from a control (stored at 6° C for 7 days) that was not inoculated. We also isolated organisms from shrimp that was held at 6° C for 7 days. Samples were inoculated in the manner previously described using 10 different bacterial species. Results indicated that *Pseudomonas fluorescens* and *Brevudimonas* sp. produced the strongest and most unpleasant odor when maintained at 6 °C for 7 days. When these bacteria were held at 30 °C for 24 hrs, there was no significant difference in spoiled shrimp odor or odor intensity compared with a control (maintained at 30° C for 24 hrs) that had not been inoculated.

## Odorous volatiles produced by selected microorganisms

In our study we determined that *Chryseomonas luteola*, *Serratia marcescens, Pseudomonas fluorescens* and *Brevudimonas* sp. were the most foul odor producing bacteria when they were allowed to grow in homogenized fresh shrimp that had been heat sterilized (endogenous enzymatic degradation and resultant odor was minimal due to inactivation by high sterilization temperatures). All four bacteria produced trimethylamine, a volatile component that is responsible for the well-known "fishy" odor associated with not-so-fresh seafood and is a result of bacterial enzymatic reduction of trimethylamine oxide, a naturally occurring constituent of fish muscle (9). Sulfur compounds were produced in abundance by each bacterium and included methanethiol (garbage

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odor), dimethyl disulfide (onion, sulfurous), thiophene (skunky), and dimethyltrisulfide (cabbage, cat urine). Isovaleric acid (cheesy, sweaty, foul foot odor) and butyric acid (baby vomit) were produced by all four bacteria. *Chryseomonas luteola* produced the most foul and intense odor. GC and gas chromatography-olfactometry analysis (Osme method) of *Chryseomonas* inoculated shrimp headspace indicated 43 aroma active compounds (25 unique to this inoculant compared to the control; Figure 2, 3) were present and the intensities for all major foul odors (trimethyl amine, sulfur compounds, short chained fatty acids) were either strongest or second strongest compared to samples inoculated with each of the other bacteria. Major differences in volatiles produced by bacteria tested were in quantities of odorants, as there did not appear to be microorganism specific production of unique odorous compounds. Indole was found in the headspace of *Enterobacter amnigenus* and *Providencia rettgeri* inoculated samples only. Because this study was not quantitative, the presence of indole in the headspace of only 2 of 17 samples tested could not be explained as a complete absence. Solid phase microextraction was utilized and its affinity for indole was not measured.

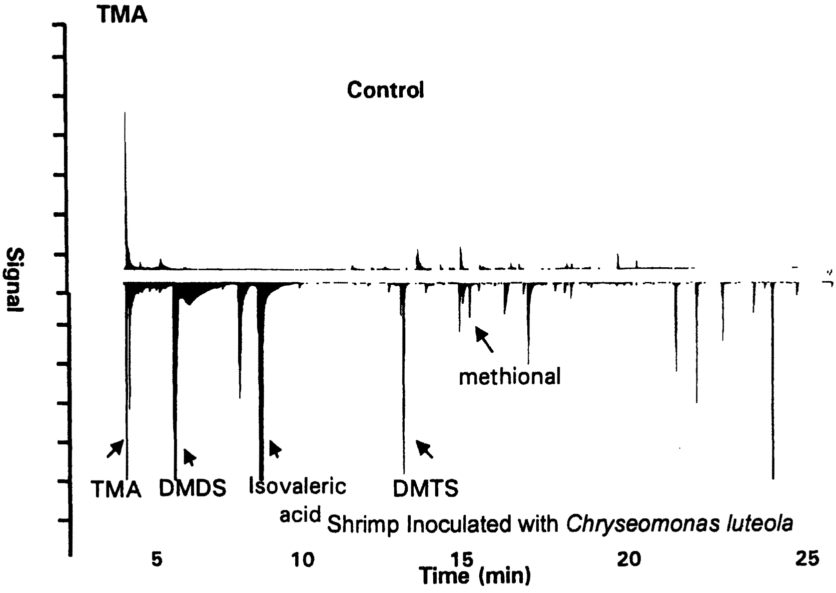

*Figure 2 GC analyses comparison: control and a sample inoculated with Chryseomonas*

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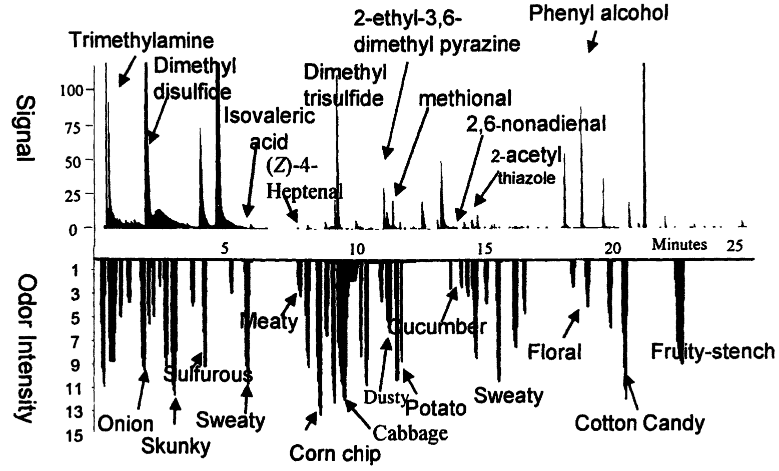

*Figure 3 Comparison of Osmegram and GC chromatogram of shrimp inoculated with Chryseomonas*

## Enzymes

Researchers have attributed the rapid onset of shrimp deterioration following harvesting to protease activity (10-16). Protease activity facilitates digestion of proteins by catalyzing peptide bond hydrolysis. Catabolism of muscle tissue is characterized by decreased firmness and decreased perception of shrimp quality. Tissue breakdown may facilitate additional deterioration and rapid spoilage by increasing availability of nutrient sources for microorganism growth. Increased volatile (odor) production may result. Sources of proteases that contribute to rapid degradation of shrimp include those endogenous to shrimp digestive processes and those produced by microorganisms present on shrimp. Yeh et al. (16) determined that adenosine deaminase , AMP deaminase, and arginase originated from shrimp tails and were involved in ammonia ($NH_3$) production. Le Boulay et al. (17) characterized cysteine protease precursors in the hepatopancreas of white shrimp. Yan et al. (18) found trypsin, chymotrypsin, and carboxypeptidase A-, and pepsin-like enzymes in the cephalothorax of red tailed shrimp and Kobatake (19) detected proteolytic activity in microorganisms isolated from Black Tiger shrimp.

Utilization of protease inhibitor to decrease the rate of shrimp degradation has potential as a shelf-life extender. Zotos and Taylor (11) demonstrated this approach when they inhibited protease isolated from Norway lobster with soybean trypsin inhibitor.

In this report we investigated the effect of trypsin and chymotrypsin on spoiled shrimp odor and odor intensity by adding 3 different concentrations of each separately to sterilized shrimp inoculated with *Chryseomonas luteola.* The concentrations of trypsin protease added were 0 units (control), 850 units , 5 x 850 units, 25 x 850 units. For chymotrypsin levels were 0 units, 150 units, 5 x 150 units, and 25 x 150 units. Concentrations added were based on the estimated amount of protease present in one shrimp (850 units for trypsin and 150 units for chymotrypsin). Results indicated that trypsin and chymotrypsin increased both spoiled shrimp odor and odor intensity linearly with increased protease concentration, and all protease treated samples were significantly different ($P<0.05$) than the inoculated, untreated control. This demonstrates that protease activity contributes to the development of spoilage odors.

As a follow on we determined the effect of protease inhibitor on spoiled shrimp odor and odor intensity. Samples were inoculated as before and treated with 3 concentrations of trypsin inhibitor (0 units, 850 units, 5 x 850 units, and 25 x 850 units). Samples were not treated with protease to ascertain if inhibition of potential *C. luteola* generated protease would affect (decrease) spoiled shrimp odor and/or odor intensity. Results indicated the inhibitor utilized had no significant effect on odor and it was concluded other proteases (other than trypsin) may be partly responsible for the odor produced.

## Freshness indicators

Freshness assessment historically has been difficult with shrimp due to a lack of rapid, reproducible testing methodology. There still is no uniform index to assess freshness or spoilage of raw shrimp product. Assessment generally employs an analysis of one or more quality attributes, including sensory, microbiological, chemical, or physical properties (20).

To encourage the current trend of expanded sales and increased popularity, shrimp processing facilities generally employ strict quality control procedures. However, the challenge is quality measurement as the product comes prepackaged with fast acting degradative microorganisms, proteolytic enzymes, and unsaturated omega-3 fatty acids that have propensities for oxidation.

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## Indole and the biogenic amines

Indole, a compound with mothball, tar odor (21) is a microorganism metabolite (L-tryptophan is the precursor) and considered an indicator of microbial activity. Currently the U.S. Food and Drug Administration (FDA) utilizes organoleptic (sensory) evaluation of shrimp odors by a trained expert, and measurement of indole levels as shrimp quality indicators (22). If sensory assessment determines samples have decomposition odors, confirmation analysis must be conducted by analyzing a minimum of six subsamples for indole (FDA website). Indole analysis is not considered a stand alone method, but is utilized in conjunction with others to provide evidence of quality (or lack of).

Liquid chromatographic (LC) and gas chromatographic (GC) procedures are delineated in AOAC for shrimp indole analysis (23). The LC procedure utilizes a fluorometric detector. The GC methodology listed in the latest AOAC (17th) edition describes utilization of a packed, glass, 6 ft GC column. Investigators have also demonstrated the effectiveness of capillary gas chromatography (24), other HPLC methodologies (25), different fluorometric detection methodologies (26), and differential-pulse voltammetry (27).

Some researchers have questioned the reliability of indole as an indicator. Niola and Maria (25) reported fresh shrimp samples had indole concentrations between 11.7 and 42.2 µg/100g, but some malodorous samples exhibited low levels of indole even after several days of storage. Solberg and Nesbakken (28) measured indole in fresh, boiled and peeled shrimp caught in the cold waters of the Barents Sea and found indole concentrations increased only in shrimp that were at an advanced stage of decomposition. They also reported some shrimp caught in warmer waters near the coasts of India, Taiwan, and Malaysia possessed high levels of indole (>25 µg/100g) but were judged organoleptically acceptable. A possible explanation for these findings may be in the origin of indole as not all microorganisms produce it. In fact, the microbes that produced the most intense and spoiled odor (*Chryseomonas luteola* and *Serratia marcescens*) in our study do not produce indole (30). Microorganisms prevalent on shrimp harvested from colder temperatures such as psychrotrophs e.g. Pseudomonads, may or may not utilize L-tryptophan as an energy source (may or may not produce indole). Bergey's manual (29) lists 5 strains of *Pseudomonas fluorescens* (one of the microorganisms we isolated) and one utilizes tryptophan, one does not, and three are indeterminate meaning they may or may not utilize it.

Other compounds purported to be useful shrimp spoilage indicators include the biogenic amines, putrescine (a volatile compound with a putrid odor, rotten odor) and cadaverine (a volatile named in honor of cadavers). Jeya et al. (30) found putrescene levels corresponded well with sensory changes. The ratio of

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total volatile nitrogen : amino acid nitrogen has been suggested as a predictor of shrimp shelf life as volatile nitrogen levels increase and amino acid nitrogen levels decrease as the product becomes degraded (31). Cobb and Vanderzant (32) found this ratio correlated well with log total plate counts as both increased at the same rate until total volatile nitrogen / amino acid nitrogen increased due to enzymic production.

## Impediometric methods

Impedance values obtained from seafood products increase with increasing storage time and protease activity, and with increased deterioration in seafood quality. This phenomenon is due to an increase in ionic metabolite products that amplify food conductivity (33). Cotton and Marshall (20) treated lobster, an organism that rapidly deteriorates following harvesting (the major reason they are cooked by boiling alive), with protease inhibitor and observed a decrease in impedance compared to the impedance of an untreated control indicating a decrease in metabolic products generated by proteolytic activity.

## Electronic nose screening and ammonia electrodes

An electronic nose is an instrument that assesses headspace volatiles with a sensor, or sensor array. The conducting polymer array may exhibit a change in electrical conductivity based on swelling characteristics caused by array and volatile compound interactions. Signals are generated due to changes in conductivity and are unique to a given volatile. A collection of unique signals forms a fingerprint for a given product, and may be utilized to discern product similarities and differences (34). Utilizing a conducting polymer electronic nose, Luzuriaga et al. (35) concluded the instrument was able to predict degrees of shrimp decomposition in a manner similar to that exhibited by an experienced sensory panel. In a separate study, hypothesizing ammonia was the main off-odor volatile component produced from shrimp, Luzuriaga et al. (36) utilized an ammonia sensor to measure headspace concentrations generated from spoiling shrimp. They reported in less than two minutes ammonia concentrations could be measured and because 230 parts per million (ppm) were deemed a rejection level for shrimp consumption by sensory panel, this device could provide a rapid and simple screening procedure for shrimp quality determination.

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## Conclusion

Shrimp spoils (develops odor, loses texture, becomes discolored) because of microorganism growth, and due to endogenous and exogenous sources of protease activity. Degradation of shrimp is accelerated with the addition of protease and it may be decreased with the addition of protease inhibitor. Sulfur compounds, short chained fatty acids, and amine containing volatiles are primarily responsible for the odor of spoiled shrimp. Differences in quantity of volatiles, rather than the presence of unique compounds, distinguishes odors between some important spoilage microorganisms.

The microorganisms present in warm and cold aquatic environments may be very different and they appear to be responsible for different spoilage patterns. The presence or absence of indicator compounds may be influenced by differences in predominant microflora.

Sensory analysis continues to be the primary screening tool for assessing shrimp quality. Analytical measurement of microorganism metabolites (indole) may provide secondary evidence of degradation, but as a stand alone method, the presence or absence of indole can not be construed as conclusive evidence for reasons previously given. A variety of techniques may permit rapid quality assessment, including electronic nose analysis and impedance measurement. Given the inherent limitations associated with chemical indicator compounds, such methodology is needed. Advances in volatile sampling (automated headspace analysis and capability for utilization of solid phase microextraction) have improved the sensitivity, accuracy, and precision of electronic nose technology. Additional studies that investigate electronic nose sensitivity to volatiles of interest (with shrimp these include amines and sulfur compounds), sensor stability over time, and ease of calibration are necessary.

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# Chapter 17

# Off-Flavor in Nile Tilapia (*Oreochromis niloticus*)

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Off-flavor causing agents in Nile Tilapia (*Oreochromis niloticus*) and various methods to mask this off-flavor are reviewed. Geosmin ($C_{12}H_{22}O$) and MIB (2-methylisoborneol) were reported as the major compounds that cause "muddy" or "musty" flavor in fish. Relationships between sensory scores and instrumental results of geosmin analysis are discussed. Similar to other fish species, geosmin can be removed from Nile tilapia by keeping it in clean static water for 16 days. Effect of various treatments i.e., salting and drying, smoking, microwave heating, marinating and fermentation on off-flavor of tilapia is also described.

In Off-Flavors in Aquaculture; Rimando, A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

# 1. Introduction

Off-flavor has been documented as a serious setback to the fish processing industry. Farm-raised fish are often rejected due to the developed offending flavor *(1)*. This may occur before as well as after the harvesting. Postharvest flavor problems are generally due to improper handling, which leads to bacterial spoilage or oxidation of fats during prolonged storage under inadequate facilities. Improving handling techniques can prevent postharvest off-flavors. However, development of off-flavors in fish before harvest is a quite complicated phenomenon where a number of contributing factors are responsible for each type of off-flavor. In pond-raised fish, off-flavors are associated with substances in the diet that are absorbed across the gastrointestinal tract and deposited in the flesh. Diet related off-flavors are described as "decay" or "rotten" and are the result of decaying organic matter in the diet. On the other hand, environment related off-flavors develop in fish, either due to accidental pollution of water such as petroleum spills from motors, boats and tanks or caused by naturally occurring aquatic microorganisms. The most common environment related off-flavors, other than those caused by pollution, are described as "earthy," "moldy," "muddy" or "musty." The muddy flavor has been reported in tilapia, carp, channel catfish, trout and a number of other fish species *(2, 3, 4)*.

Geosmin, synthesized by species of blue-green algae and actinomycetes and excreted into the environment, were found responsible for this off-flavor in channel catfish *(5)*. Geosmin can be absorbed through the gills of fish from water and through the digestive tract, eventually being deposited in fat tissues. This affects its flavor *(6)*. A number of schemes have been proposed to manage off-flavor due to aquatic microorganisms, but most approaches have either been impractical or unreliable. Moreover, each off-flavoring compound originates from a different unique source which is often unknown. It is therefore impossible to eliminate off-flavor with a single management strategy. Elimination of the off-flavor causing microorganisms is possible by using growth inhibitory chemicals, e.g., algicides for blue-green algae. However, using chemicals also presents a number of problems: different growth inhibitors for each kind of microorganism, temperature fluctuations affect the performance of chemicals, environmental issues and economical concerns. Once the off-flavoring compound is deposited in fish, the deposited geosmin content can be reduced by transferring fish from its natural habitat to a clean pond water but this is not an economically feasible method.

Tilapia (*Oreochromis niloticus*) originate in the Nile river and have been found to be suitable for farm raising due to its high growth rate and short cultural period *(7)*. It is a mild sweet tasting fish with a slightly firm and flaky

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texture. Its tolerant nature to a wide range of environmental conditions have led to its distribution worldwide. Tilapia is claimed to be the most cultured fish after carp *(8)*. Being omnivorous, tilapia mainly feeds on phytoplankton which results in development of off-flavor due to accumulation of geosmin content in its flesh. Similarly to other fish genera, the tilapia market is also being greatly affected by the unpleasant taste.

In addition to off-flavor problems of fish, their perishable nature necessitated the need for preservation techniques which had been developed according to climatic and geographical conditions of various regions. In recent years, the effect of conventional as well as modern techniques and their combinations to mask the off-flavor of fish have been studied. The preservation methods such as salting, drying, smoking, frying, microwave heating, marinating and fermentation with carbohydrate mixture extend the shelf life of fish and mask or remove the muddy-musty or earthy flavor as well. Fish salting with subsequent drying imparts a desirable flavor to fish *(9)*. Preservation of rainbow trout by smoking and by partial cooking was attempted *(10)*. Vegetable oil can also be added before canning to eliminate earthy-musty flavor. The physical properties of fish flesh have been found to be suitable for microwave heating to enable removal of off-flavor. In the process called marinating, acetic acid and salt mixtures are applied to preserve tilapia. Lactic acid fermentation has also been a traditional fish preservation technique, and a controlled assay of such fermentation has great potential to improve the shelf life of Nile tilapia with a more acceptable form to consumers. However, little work is known on lactic acid fermentation effects on reducing off-flavor in fish. These processing schemes may mask the off-flavor that was not possible to eliminate completely at the farm level. By implementing these techniques, both farmers and consumers reap benefits, and consumers may enjoy a nutritious as well as an on-flavored product.

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## 2. Cause of Muddy Flavor in Fish

Environment-related off-flavor was first reported in 1936 *(11)*. It was noticed that salmon that came from a stream in England were unpalatable due to a strong earthy flavor. The cause of this earthy flavor was found to be the Actinomycetes growing in the river bank *(12)*. Several other researchers also reported that blue-green algae and odor producing actinomycetes were responsible for "earthy-musty" flavor in different fish species *(4, 10, 13, 14)*. In the tropics, milkfish and tilapia were reported to bear a typical flavor named by researchers as muddy flavor *(15, 16)*.

Odors produced by actinomycetes were noted to be similar to those of freshly ploughed soil *(17)*. Over several years of research, a compound was isolated from *Streptomyces orlseus* culture, identified as *trans*-1,10-dimethyl-*trans*-9-decalol and named geosmin *(18, 19)*. Later, another compound, 2-methylisoborneol (MIB), was also isolated and it smells like camphor in high concentrations but has a muddy odor in diluted concentrations *(20, 21)*.

Geosmin has four isomers, but not all are responsible for off-flavor *(22)*. It is a metabolite of many species of actinomycetes *(18)* and several algae *(23)*, such as *Aphanizomenon* sp. *(24)* and *Anabaena circinalis (25)*. Geosmin has been isolated from a number of fish species such as rainbow trout *(4)*, channel catfish *(26)*, shrimp *(27)*, carp and bream *(28)*. Odorous actinomycetes were found in waters, but no clear relationship between actinomycetes and muddy odor was found *(16)*. In the integrated polyculture pond of tilapia, it was found that off-flavor fish usually occurred in ponds of low transparency *(16)*. Off-flavor in fish is also directly related to feeding rates *(6)*.

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## 2.1 Chemical Characteristics of Geosmin and MIB

Geosmin and MIB are described as colorless, neutral oils which darken very slightly after long storage and are unstable in acidic environments *(19)*. Geosmin is a volatile metabolite compound, identified as *trans*-1,10-dimethyl-*trans*-9-decalol, $C_{12}H_{22}O$ *(18)*. It's a neutral oil, with an approximate boiling point of 270°C and contains 79% carbon and 12% hydrogen. Geosmin and MIB are both saturated tertiary alcohols and therefore, stable to oxidation. With acid treatment, geosmin changes into an odorless compound argosmin which is composed of 86% carbon and 12% hydrogen.

## 2.2 Absorption of Geosmin

Geosmin and other off-flavor causing compounds are believed to be absorbed through the gills, alimentary canal and skins *(5, 6, 29, 30)*. Water, including dissolved compounds and ions, are absorbed through gills and excess water in the body excreted through the kidneys due to osmoregulation. Therefore, the compounds are distributed in the body through the circulatory system. It was noted that unlike live fish, dead fish do not absorb dissolved compounds from water *(11)*. Later, it was confirmed that absorption of flavoring compounds is independent of the digestive tract and probably occurs through the gills into the blood stream *(14)*. During a set of experiments, channel catfish were placed in water tanks containing unialgal cultures of geosmin-producing blue-green algae. The muddy flavor developed in less than 24 hours and became stronger in two days. In contrast to this, when fish were placed in filtered water, the muddy flavor developed more slowly *(16)*.

However, absorption is affected by a number of factors, such as the concentration of compounds, exposure time, synergistic effects between compounds, species of fish and water temperature *(17)*.

## 2.3 Geosmin Distribution in Tilapia

The amount of geosmin content in different body parts of fish can be measured by various methods (which will be discussed in the following section). The intestine of tilapia posseses the highest concentration of geosmin *(16)*. Geosmin in intestines and other parts of the body was found in the following order: intestines (741 ug/kg), abdominal tissues (327 ug/kg), skin (315.5 ug/kg) and flesh (240.8 ug/kg) *(16)*.

## 2.4 Geosmin Analysis and Flavor Intensity

Subjectively, geosmin content can be estimated in terms of off-flavor. An expert sensory panel is employed to detect flavor intensity. A scoring system has been proposed which ranges from threshold (0), very slight (0.5), slight (1), slight to moderate (1.5), moderate (2), moderate to strong (2.5) and strong (3) *(31)*. The results of sensory analysis are usually correlated to the chemical or physical properties of fish. However, an objective method such as GC analysis for direct estimation of geosmin content is much preferred due to high accuracy and no risk of human errors. This involves distillation of geosmin from the sample, concentration of distillate and analysis by gas chromatography *(4)*. A high correlation was found between sensory scores for off-flavor and instrumental analysis of geosmin in various studies *(32)*. A logarithmic relationship with $R^2$ of 0.727 between sensory and instrumental scores of geosmin analysis in tilapia was found. The latter result was in accordance to the Weber-Fechner Law which states that sensory response and chemical concentration are proportionally based upon logarithmic regression *(33)*. It was further found that the relationship between geosmin concentration and threshold odor number in water seemed to be appropriately explained by the Stevens' log-log model *(34)*. Therefore, it is concluded that instrumental analysis data can be used to quantify flavor intensity (threshold levels) of the fish.

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## 2.5 Removal of Geosmin

Purging of geosmin is done by transferring fish into static, clean water. Partial water exchange is made daily to maintain water quality. However, the rate of removal of muddy flavor is reported to be much slower than the uptake, and the fish are expected to purge geosmin content in more than 5 days *(35, 36)*. Temperature of the water also plays a significant role for geosmin purging. The rate of purging was decreased when the temperature of the water was lower than 22°C *(6)*. The purging time for geosmin in tilapia also depends on the amount of geosmin absorbed. It was observed that geosmin content was removed from different parts of the body in 16 days when fish were kept in static clean water *(37)*. The geosmin level rapidly decreased during the first 4 days, especially in the intestines. This was probably due to the lower geosmin concentrations in other parts of the body such as skin, flesh and abdominal tissues. Experiments conducted on channel catfish *(14)* and rainbow trout *(12)* also showed that at least 16 days were required to flush out geosmin below detectable levels (i.e., <0.1 ug/kg of fish).

# 3. Preservation Methods and Off-flavor

As described earlier, a number of methods can be used to mask or reduce the muddy flavor such as salting, drying, smoking, frying, microwave heating, marinating and fermentation with carbohydrate mixture. An overview on applications of these methods to Nile tilapia and their effects on its quality will be discussed in the following sections.

## 3.1 Salting and Drying

Fish may be salted utilizing three methods: kench curing, pickling and brining *(38)*. Kench curing is rubbing the added salt with fish flesh. With the penetrating action of salt into the flesh, water drains off during stacking. In the case of pickling, the moisture extracted out of the flesh is not allowed to drain. Brining is soaking of fish in concentrated salt solution. Under tropical conditions, brining and pickling are much preferred over kench curing as both help even salting. Covering fish with salt helps to protect it from halophilic bacteria, insects and rancidity *(39)*.

The salt uptake by fish depends on various factors such as fat content of the fish, thickness of the fish and concentration of the salt *(40)*. The higher the fat content of the fish, the slower the salt up take. Similarly, salt diffusion will

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be slower in thick fish flesh. The concentration of salt at the center of a fillet of 2.5 cm thickness may reach 10% after 24 hours in salt. It may take 3 days to reach the same concentration for a fillet of the same fish that is 5 cm thick.

Salt is applied to Nile tilapia in both wet and dried forms *(16)*. In the wet salting process, the dressed fish is soaked in a saturated brine solution (26% w/v) for 2 hours (fish to salt ratio 2:1). A fish to salt ratio of 5:1 is chosen to apply in the dry form for 4 hours. After rinsing and cleaning, the fish is allowed to dry in the sun. The geosmin content in the salted and dried fish has been found to be 17.4-19.8 ug/kg when the initial level of geosmin in the fish was 21.9 ug/kg *(16)*. Because it is a traditional technique of preserving fish and there is variability in environmental conditions, no standards for salting and subsequent drying of fish have been established. For instance, in the tropics, salted fish is dried up to 50-55% final moisture content *(16)*. The salted fish is usually dried under the sun, however, researchers have compared sun drying with hot-air oven drying at temperatures of 40°C, 50°C and 60°C. There was no significant difference between both drying methods in terms of sensory quality, however, hot-air oven drying could be used with high reliability as a controlled and hygienic way of drying. It was also found that the use of high temperatures could result in poor quality fish *(37)*.

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### 3.2 Smoking

Smoking is conducted in four different ways: cold smoking (<30°C), hot smoking (70-80°C), liquid smoking (liquid used to absorb smoke and then concentrated) and electrostatic smoking (high voltage field). Usually a hardwood is used for smoking. However, the choice of wood depends upon the choice of smoking sources and the flavor desired in the final product *(42)*. The type of wood, relative humidity, temperature, smoke velocity, smoke density and air flow are the main factors affecting the quality of the final product *(9)*. During smoke drying, the fish gradually lose weight as water is lost. Lowering water content gives higher preservation ability to the product, but makes the meat tough and reduces the yield *(42)*.

Cold smoking imparts flavor of the preservative while hot smoking can cause case hardening. A combination of cold and hot smoking is suggested *(16, 42)*. Fish samples can be smoked for 5 hours, i.e., 40°C for 4 hours and 60°C for the last hour. The first step of heating effectively removes excess water from the surface and makes the smoke drying feasible. In the second step, the fish is cooked.

### 3.3 Microwave Heating

Moisture and salt content of the material determine the uniformity of heating when microwave heating is applied to the materials. The greater the moisture and salt content, the shallower the microwave penetration depth and, consequently, the less uniform heating rate throughout the product. Microwave heating causes moisture migration in food and may influence the off-flavor. However, little work has been done to study the effect of microwave heating on muddy flavor of fish. A study conducted on microwave effects on off-flavor showed that microwaves did not affect the flavor of either muddy or non muddy flavored tilapia. It was further reported that geosmin analysis of both muddy and non-muddy flavored fish before and after microwave treatment showed no significant change, i.e., 20.89-21.66 ug/kg and 8.0-8.49 ug/kg, repectively *(37)*.

### 3.4 Marinade Treatments

Marinating refers to keeping fish in a mixture of acetic acid and salt to extend its shelf life with a characteristic flavor *(42)*. The action of proteolytic enzymes along with acetic acid causes tenderness to the flesh and, consequently, the breakdown of the proteins and release of free amino acids. Marinades may be applied in three different ways: cold marinades, cooked marinades and fried marinades *(43)*. In cold marinades, fish fillets are immersed in 7% acetic acid and 1.5% salt solution. No heating is applied in cold marinades. Cooked marinades are prepared by keeping the fish fillets in the same solution of salt and acid at 85°C for 15 minutes, while in the case of fried marinades, fish fillets are fried at 160-165°C for 10 minutes before immersing in salt-acid mixture. Cold marinade was not found effective to remove the muddy flavor as compared to cooked and fried marinades. Geosmin content was found to decrease from an initial value of 21 ug/kg to 15.4 ug/kg in cooked marinade and to 8 ug/kg for fried marinade, while a very slight change was observed in geosmin content for the cold marinade during 14 days of marinating *(37)*. The reduction in geosmin content in the fried marinated product was attributed to loss of water along with geosmin during frying *(43)*. It was also suggested that due to acetic acid fermentation, substantial extraction of fresh fish volatile carbonyls *(44)* and degradation of geosmin compounds into odorless argosmin compounds could occur *(18, 45)*. Longer periods under marinating further decrease the geosmin content *(37)* as the penetrating action of acetic acid into the flesh of fish continues. It has also been reported that no muddy flavor was detected after 24 days following fried marinating *(37)*. A shelf life of 1, 6 and 12 months for cold, cooked and fried marinades, respectively, is expected *(43)*.

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### 3.5 Carbohydrate Fermentation (*Som fak*)

In Thailand, a traditional fish preservation technique, called *som fak*, has been found to be very effective for masking muddy flavor in tilapia *(37)*. To prepare *som fak*, minced fish is combined with 15% boiled rice, 4% salt and minced garlic and then mixed thouroughly, kneaded into an elastic firm mass, packed in banana leaves and allowed to ferment. In the laboratory prepared samples, an initial 8.6 ug/kg geosmin content was decreased to 1.45 ug/kg following 20 days of storage *(37)*. The reduction in geosmin content is due to the action of lactic acid produced during fermentation which degrades geosmin into argosmin compounds *(18, 45)*.

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## 4. Summary

Fish readily absorb certain compounds from the environment and from their diet. This results in off-flavor if the diet contains certain types of organic matter, the habitat is polluted or it contains certain types of phytoplankton species. Diet and pollution related off-flavors can be prevented by effective management, but postharvest off-flavor may occur due to microorganisms and this presents a number of problems for the industry. Geosmin and MIB are the compounds found to be responsible for the typical muddy flavor that has been reported in a number of fish species, especially Nile tilapia. The concentration of the compounds in fish samples can be estimated by vacuum distillation, extraction and gas chromatography. Geosmin distribution in tilapia was reported to be the highest in the intestine and in decreasing order in the abdomen, skin and muscle tissues.

Off-flavored tilapia and its products can be successfully marketed by applying certain preservation and processing techniques: salting and drying, smoking with pre-treatments, deep frying, soaking in acetic acid environment and by lactic acid fermentation *(som fak)*. Among cooking methods, microwave heating has no significant effect on geosmin levels in tilapia.

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